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OF
EXPERIMENTAL MEDICINE

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VOLUME 147
1978

PUBLISHED MONTHLY BY
THE ROCKEFELLER UNIVERSITY PRESS

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CONTENTS

Volume 147 No. 1 January 1, 1978

1 Michael P. Cancro, Nolan H. Sigal, and Norman R. Klinman. Differential expression of an equivalent clonotype among BALB/c and C57BL/6 mice

13 Eugene E. Emeson. Migratory behavior of lymphocytes with specific reactivity to alloantigens. II. Selective recruitment to lymphoid cell allografts and their draining lymph nodes

25 Peter H. Krammer. Alloantigen receptors on activated T cells in mice. I. Binding of alloantigens and anti-idiotypic antibodies to the same receptors

39 James Watson, Michael Largen, and Keith P. W. J. McAdam. Genetic control of endotoxic responses in mice

50 M. Aguet, L. C. Andersson, R. Andersson, E. Wight, H. Binz, and H. Wigzell. Induction of specific immune unresponsiveness with purified mixed leukocyte culture-activated T lymphoblasts as autoimmunogen. II. An analysis of the effects measured at the cellular and serological levels

63 Hans Binz and Hans Wigzell. Induction of specific immune unresponsiveness with purified mixed leukocyte culture-activated T lymphoblasts as autoimmunogen. III. Proof for the existence of autoanti-idiotypic killer T cells and transfer of suppression to normal syngeneic recipients by T or B lymphocytes

77 Paul J. Edelson and Cheryl Erbs. Plasma membrane localization and metabolism of alkaline phosphodiesterase I in mouse peritoneal macrophages

87 C. E. Calkins and O. Stutman. Changes in suppressor mechanisms during postnatal development in mice

98 Stephen R. Holdsworth, Napier M. Thomson, Eric F. Glasgow, John P. Dowling, and Robert C. Atkins. Tissue culture of isolated glomeruli in experimental crescentic glomerulonephritis

110 Sirkka Kontiainen and Marc Feldmann. Suppressor-cell induction in vitro. IV. Target of antigen-specific suppressor factor and its genetic relationships

123 Ronald N. Germain, Jacques Thèze, Judith A. Kapp, and Baruj Benacerraf. Antigen-specific T-cell-mediated suppression. I. Induction of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} specific suppressor T cells in vitro requires both antigen-specific T-cell-suppressor factor and antigen

137 E. G. Engleman, A. J. McMichael, M. E. Batey, and H. O. McDevitt. A suppressor T cell of the mixed lymphocyte reaction in man specific for the stimulating alloantigen. Evidence that identity at HLA-D between suppressor and responder is required for suppression

147 F. J. Ramalho-Pinto, Diane J. McLaren, and S. R. Smithers. Complement-mediated killing of schistosomula of *Schistosoma mansoni* by rat eosinophils in vitro

157 Henry Masur and Thomas C. Jones. The interaction in vitro of *Pneumocystis carinii* with macrophages and L-cells

171 Alan B. Wagshal, Brian V. Jegasothy, and Byron H. Waksman. Regulatory substances produced by lymphocytes. VI. Cell cycle specificity of inhibitor of DNA synthesis action in lymphocytes

182 Gary E. Hatch, Donald E. Gardner, and Daniel B. Menzel. Chemiluminescence of phagocytic cells caused by *N*-formylmethionyl peptides

196 David H. Sherr, Myron R. Szewczuk, and Gregory W. Siskind. Ontogeny of B-lymphocyte function. V. Thymus cell involvement in the functional maturation of B-lymphocytes from fetal mice transferred into adult irradiated hosts

207 M. H. Ginsberg and P. M. Henson. Enhancement of platelet response to immune complexes and IgG aggregates by lipid A-rich bacterial lipopolysaccharides

219 Stephen J. O'Brien and Janice M. Simonson. *Bvr-1*, a restriction locus of a type C RNA virus in the feline cellular genome: pleiotropic restriction of endogenous BALB virus in cat × mouse somatic cell hybrids

233 Hartmut Wekerle. Immunological T-cell memory in the in vitro-induced experimental autoimmune orchitis. Specificity of the reaction and tissue distribution of the autoantigens

recipients prepared with TLI showed no clinical signs of GVHD, and 85% survived longer than 250 days. The surviving animals were stable chimeras. Recipients given thymic irradiation showed no evidence of marrow engraftment.

Materials and Methods

Animals. Inbred BALB/c (H-2$^{d/d}$) mice, 4–6 mo old, were used as recipients for skin and BM allografts. Inbred C57BL/Ka (H-2$^{b/b}$), 1–4 mo old, were the donors of BM and skin grafts. All mice were bred in pathogen-free conditions in the Division of Radiobiology, Department of Radiology, Stanford University, but they were transferred to conventional housing before use in irradiation experiments. At the onset of irradiation, a broad-spectrum antibiotic (tetracycline hydrochloride; American Cyanamid Co., Princeton, N. J.) was added to the drinking water.

Radiation Source. A Philips unit (250 kV, 15 mA; Philips Medical Systems Inc., Shelton, Conn.) delivered x-rays at a rate of 59 rad/min. The source-to-skin distance was 60 cm. 0.25-mm Cu and 1.0-mm Al correction filters were used and the dosimetry was determined using a calibrating ionizing chamber and lithium fluoride thermoluminescence dosimeters.

Irradiation Procedure. The irradiation procedure was previously described in detail (9, 11, 12). Anesthetized BALB/c mice were positioned in a lead apparatus, exposing the major lymphoid organs, including the thymus and the spleen with proper shielding of the skull, lungs, ribs, hind legs, and tail. In some experiments, the thymus was shielded by placing a piece of lead (0.5 x 0.5 cm) over the mediastinum. Thymic irradiation alone was accomplished by exposing the mediastinum, and shielding the rest of the body with lead. Irradiation protocols consisted of fractions of 200 rads/day, five times a week, to achieve a total dose of 3,400 rads (unless specified otherwise in the text). WBI was delivered using the same radiation source to administer a single dose of 1,000 rads.

BM Transplantation. The BM cells were flushed out of isolated long bones (femur, tibia, and humerus) using a 25-gauge needle, and minimal essential medium (MEM). The cells were washed once, filtered through a nylon mesh, and resuspended in MEM. 0.25-ml aliquots of medium containing 10×10^6 or 30×10^6 nucleated cells were injected into the lateral tail vein of recipient mice.

Skin Transplantation. Full-thickness C57BL/Ka skin obtained from the abdomen of 4- to 6-wk-old female mice were transplanted to the flank of BALB/c recipients (13). Grafts were considered rejected at the time of complete sloughing, or when a dry scab was formed.

Purification of Peripheral Blood Lymphocytes (PBL). Blood obtained from the retro-orbital veins was placed in preservative-free heparin coated glass tubes. Samples were diluted (1:5) in phosphate-buffered saline (PBS), and the PBL were purified using a Ficoll-Hypaque gradient (14). Contaminating erythrocytes were lysed with ammonium chloride (15).

Assay for Chimerism. Chimerism of the PBL of BM recipients was assayed using a complement-dependent microcytotoxicity assay with a specific anti-H-2^b alloantiserum (B10.A(5R) × A)F$_1$ anti-B10.A(2R) (kindly supplied by Dr. D. B. Murphy, Department of Medicine, Stanford University) as described previously (13). Purified PBL were suspended in medium 199 (Microbiological Associates, Inc., Bethesda, Md.) containing 5% heat-inactivated fetal calf serum (2×10^6 cells/ml). 10 μl of the lymphocyte suspension was incubated with 10 μl of anti-H-2^b antiserum (1:10) for 30 min at room temperature in 6 x 50-mm glass tubes (Kimble Products, Div. Owens-Illinois, Inc., Toledo, Ohio). 5 μl of guinea pig complement was added for an additional 45 min at room temperature. The cells were spun at 250 *g* for 5 min, and resuspended in 15 μl of medium 199. 15 μl of a 0.1% solution of trypan blue was added before examining the viability of cells in a standard hemocytometer. The cytotoxicity index was calculated by comparing the number of viable cells present after treatment with anti-H-2^b antiserum to that after treatment with normal mouse serum. Negative (BALB/c; PBL $\leq$5% cytotoxicity) and positive (C57BL/Ka; PBL, 95–100% cytotoxicity) controls were performed in each experiment. Cytotoxicity assays were done in triplicate, and read by a blind observer. Data shown in tables (percent of donor [H-2^b] type cells) is the mean ± SD of triplicate values of the cytotoxicity index.

Results

Effect of TLI on Allogeneic Skin and Marrow Survival. Adult BALB/c mice were given marrow and/or skin allografts 1 day after the completion of TLI. TLI

as a result of radiation leukemia virus infection: a possible mechanism for *H-2*-linked control of virus-induced neoplasia

488 Russell E. Thompson, Robert Mandle, Jr., and Allen P. Kaplan. Characterization of human high molecular weight kininogen. Procoagulant activity associated with the light chain of kinin-free high molecular weight kininogen

500 Hannes Frischknecht, Hans Binz, and Hans Wigzell. Induction of specific transplantation immune reactions using anti-idiotypic antibodies

515 K-P. Chang and D. M. Dwyer. *Leishmania donovani*. Hamster macrophage interactions in vitro: cell entry, intracellular survival, and multiplication of amastigotes

531 Jean Lindenmann, Esther Deuel, Sergio Fanconi, and Otto Haller. Inborn resistance of mice to myxoviruses: macrophages express phenotype in vitro

541 Lutz H. Block, Herbert Jaksche, Stephan Bamberger, and Gerhard Ruhenstroth-Bauer. Human migration inhibitory factor: purification and immunochemical characterization

554 James E. Swierkosz, Kenneth Rock, Philippa Marrack, and John W. Kappler. The role of *H-2*-linked genes in helper T-cell function. II. Isolation on antigen-pulsed macrophages of two separate populations of F_1 helper T cells each specific for antigen and one set of parental *H-2* products

571 Ronald P. Daniele, Sandra K. Holian, and Peter C. Nowell. A potassium ionophore (nigericin) inhibits stimulation of human lymphocytes by mitogens

BRIEF DEFINITIVE REPORTS

582 Robert A. Eisenberg, Eng M. Tan, and Frank J. Dixon. Presence of anti-Sm reactivity in autoimmune mouse strains

588 Eva Orlans, Jane Peppard, John Reynolds, and Joe Hall. Rapid active transport of immunoglobulin A from blood to bile

593 S. I. Hamburg, R. E. Manejias, and M. Rabinovitch. Macrophage activation: increased ingestion of IgG-coated erythrocytes after administration of interferon inducers to mice

599 Beverly D. Deak, Daniel Meruelo, and Hugh O. McDevitt. Expression of a single major histocompatibility complex locus controls the immune response to poly-L-(tyrosine, glutamic acid)-poly-DL-alanine-poly-L-lysine

605 Robert L. Evans, Leonard Chess, Herbert Levine, and Stuart F. Schlossman. Antibody-dependent cellular cytotoxicity by allosensitized human T cells

611 Ian F. C. McKenzie and Margaret M. Henning. The differential destructive and enhancing effects of anti-H-2k, H-2D, and anti-Ia antisera on murine skin allografts

Volume 147 No. 3 March 1, 1978

619 Carl E. Frasch and Joan D. Robbins. Protection against group B meningococcal disease. II. Infection and resulting immunity in a guinea pig model

629 Carl E. Frasch and Joan D. Robbins. Protection against group B meningococcal disease. III. Immunogenicity of serotype 2 vaccines and specificity of protection in a guinea pig model

645 Carmen Fernandez and Göran Möller. Immunological unresponsiveness to native dextran B512 in young animals of dextran high responder strains is due to lack of Ig receptor expression. Evidence for a nonrandom expression of V-genes

656 Michel Pierres, Ronald N. Germain, Martin E. Dorf, and Baruj Benacerraf. In vivo effects of anti-Ia alloantisera. I. Elimination of specific suppression by in vivo administration of antisera specific for I-J controlled determinants

667 Ammon B. Peck, Robert A. Murgita, and Hans Wigzell. Cellular and genetic restrictions in the immunoregulatory activity of alpha-fetoprotein. I. Selective inhibition of anti-Ia-associated proliferative reactions

684 Woodruff Emlen and Mart Mannik. Kinetics and mechanisms for removal of circulating single-stranded DNA in mice

700 S. Slavin, B. Reitz, C. P. Bieber, H. S. Kaplan, and S. Strober. Transplantation

tolerance in adult rats using total lymphoid irradiation: permanent survival of skin, heart, and marrow allografts

708 Nelson H. Pazmiño, James N. Ihle, and Allan L. Goldstein. Induction in vivo and in vitro of terminal deoxynucleotidyl transferase by thymosin in bone marrow cells from athymic mice

719 Susan D. Revak, Charles G. Cochrane, Bonno N. Bouma, and John H. Griffin. Surface and fluid phase activities of two forms of activated Hageman factor produced during contact activation of plasma

730 Gordon D. Ross, Charles I. Jarowski, Enrique M. Rabellino, and Robert J. Winchester. The sequential appearance of Ia-like antigens and two different complement receptors during the maturation of human neutrophils

745 Keld Danø and E. Reich. Serine enzymes released by cultured neoplastic cells

758 M. Hurme, C. M. Hetherington, P. R. Chandler, and Elizabeth Simpson. Cytotoxic T-cell responses to H-Y: mapping of the Ir genes

768 M. Hurme, P. R. Chandler, C. M. Hetherington, and Elizabeth Simpson. Cytotoxic T-cell responses to H-Y: correlation with the rejection of syngeneic male skin grafts

776 Michael P. Cancro, Walter Gerhard, and Norman R. Klinman. The diversity of the influenza-specific primary B-cell repertoire in BALB/c mice

788 Stephen D. Miller, Man-Sun Sy, and Henry N. Claman. Genetic restrictions for the induction of suppressor T cells by hapten-modified lymphoid cells in tolerance to 1-fluoro-2,4-dinitrobenzene contact sensitivity. Role of the *H-2D* region of the major histocompatibility complex

800 Michael G. Goodman, D. Elliot Parks, and William O. Weigle. Immunologic responsiveness of the C3H/HeJ mouse: differential ability of butanol-extracted lipopolysaccharide (LPS) to evoke LPS-mediated effects

814 Donna G. Sieckmann, Richard Asofsky, Donald E. Mosier, Ian M. Zitron, and William E. Paul. Activation of mouse lymphocytes by anti-immunoglobulin. I. Parameters of the proliferative response

830 Edward C. Kisailus and Elvin A. Kabat. Immunochemical studies on blood groups. LXVI. Competitive binding assays of A_1 and A_2 blood group substances with insolubilized anti-A serum and insolubilized A agglutinin from *Dolichos biflorus*

844 Richard M. Bartholomew, Alfred F. Esser, and Hans J. Müller-Eberhard. Lysis of oncornaviruses by human serum. Isolation of the viral complement (C1) receptor and identification as p15E

854 Syamal K. Datta, Noga Manny, Chester Andrzejewski, Janine André-Schwartz, and Robert S. Schwartz. Genetic studies on autoimmunity and retrovirus expression in crosses of New Zealand black mice. I. Xenotropic virus

872 Syamal K. Datta, Patricia J. McConahey, Noga Manny, Argyrios N. Theofilopoulos, Frank J. Dixon, and Robert S. Schwartz. Genetic studies of autoimmunity and retrovirus expression in crosses of New Zealand black mice. II. The viral envelope glycoprotein gp70

882 R. M. Zinkernagel, G. N. Callahan, A. Althage, S. Cooper, P. A. Klein, and J. Klein. On the thymus in the differentiation of "H-2 self-recognition" by T cells: evidence for dual recognition?

897 R. M. Zinkernagel, G. N. Callahan, A. Althage, S. Cooper, J. W. Streilein, and J. Klein. The lymphoreticular system in triggering virus plus self-specific cytotoxic T cells: evidence for T help

912 S. K. Lee and R. T. D. Oliver. Autologous leukemia-specific T-cell-mediated lymphocytotoxicity in patients with acute myelogenous leukemia

923 N. N. Iscove and F. Melchers. Complete replacement of serum by albumin, transferrin, and soybean lipid in cultures of lipopolysaccharide-reactive B lymphocytes

BRIEF DEFINITIVE REPORTS

934 Isabel Lemaître-Coelho, Graham Douglas Fisher Jackson, and Jean-Pierre Vaerman. High levels of secretory IgA and free secretory component in the serum of rats with bile duct obstruction

940 D. Volf, L. L. Sensenbrenner, S. J. Sharkis, G. J. Elfenbein, and I. Scher. Induction of partial chimerism in nonirradiated B-lymphocyte-deficient CBA/N mice

946 Matthew Mescher, Linda Sherman, Francois Lemonnier, and Steven Burakoff. The induction of secondary cytolytic T lymphocytes by solubilized membrane proteins

952 J. I. Kurland and R. Bockman. Prostaglandin E production by human blood monocytes and mouse peritoneal macrophages

959 Correction

Volume 147 No. 4 April 1, 1978

963 S. Slavin, Z. Fuks, H. S. Kaplan, and S. Strober. Transplantation of allogeneic bone marrow without graft versus host disease using total lymphoid irradiation

973 Vanda A. Lennon, Marjorie E. Seybold, Jon M. Lindstrom, Charles Cochrane, and Richard Ulevitch. Role of complement in the pathogenesis of experimental autoimmune myasthenia gravis

984 Samuel J. Black, Wessel van der Loo, Michael R. Loken, and Leonard A. Herzenberg. Expression of IgD by murine lymphocytes. Loss of surface IgD indicates maturation of memory B cells

997 Judith A. Kapp. Immunosuppressive factors from lymphoid cells of nonresponder mice primed with L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}. IV. Lack of strain restrictions among allogeneic, nonresponder donors and recipients

1007 Charles E. Davis, Elizabeth J. Ziegler, and Karen F. Arnold. Neutralization of meningococcal endotoxin by antibody to core glycolipid

1018 Margaret A. Alspaugh, Fred C. Jensen, Harvey Rabin, and Eng M. Tan. Lymphocytes transformed by Epstein-Barr virus. Induction of nuclear antigen reactive with antibody in rheumatoid arthritis

1028 Kenji Okuda and Chella S. David. A new lymphocyte-activating determinant locus expressed on T cells, and mapping in *I-C* subregion

1037 Edgar G. Engleman, Andrew J. McMichael, and Hugh O. McDevitt. Suppression of the mixed lymphocyte reaction in man by a soluble T-cell factor. Specificity of the factor for both responder and stimulator

1044 Charles D. Scher. Effect of pseudotype on Abelson virus and Kirsten sarcoma virus-induced leukemia

1054 Svante Stenman and Antti Vaheri. Distribution of a major connective tissue protein, fibronectin, in normal human tissues

1065 Charles A. Janeway, Jr., Patricia D. Murphy, John Kemp, and Hans Wigzell. T cells specific for hapten-modified self are precommitted for self major histocompatibility complex antigens before encounter with the hapten

1078 Edward F. Voelkel, Lawrence Levine, Chester A. Alper, and Armen H. Tashjian, Jr. Acute phase reactants ceruloplasmin and haptoglobin and their relationship to plasma prostaglandins in rabbits bearing the VX$_2$ carcinoma

1089 Yuichi Obata, Elisabeth Stockert, Paul V. O'Donnell, Shuji Okubo, Harry W. Snyder, Jr., and Lloyd J. Old. $G_{(RADA1)}$: a new cell surface antigen of mouse leukemia defined by naturally occurring antibody and its relationship to murine leukemia virus

1106 D. D. Eardley, J. Hugenberger, L. McVay-Boudreau, F. W. Shen, R. K. Gershon, and H. Cantor. Immunoregulatory circuits among T-cell sets. I. T-helper cells induce other T-cell sets to exert feedback inhibition

1116 H. Cantor, L. McVay-Boudreau, J. Hugenberger, K. Naidorf, F. W. Shen, and R. K. Gershon. Immunoregulatory circuits among T-cell sets. II. Physiologic role of feedback inhibition in vivo: absence in NZB mice

1126 Naomi Rosenberg and David Baltimore. The effect of helper virus on Abelson virus-induced transformation of lymphoid cells

1142 J. Sprent. Restricted helper function of F$_1$ hybrid T cells positively selected to heterologous erythrocytes in irradiated parental strain mice. I. Failure to collaborate with B cells of the opposite parental strain not associated with active suppression

1159 J. Sprent. Restricted helper function of F_1 hybrid T cells positively selected to heterologous erythrocytes in irradiated parental strain mice. II. Evidence for restrictions affecting helper cell induction and T-B collaboration, both mapping to the *K*-end of the *H-2* complex

1175 Saimon Gordon and Zanvil A. Cohn. Bacille Calmette-Guérin infection in the mouse. Regulation of macrophage plasminogen activator by T lymphocytes and specific antigen

1189 Mary A. Maloney, Mary J. Dorie, Rosito A. Lamela, Zora R. Rogers, and Harvey M. Patt. Hematopoietic stem cell regulatory volumes as revealed in studies of the bg^J/bg^J: W/W^v chimera

1198 Terry L. Delovitch, Judith Biggin, and Foon-Yee Fung. In vitro analysis of allogeneic lymphocyte interaction. II. *I*-region control of the activity of a B-cell-derived *H-2*-restricted allogeneic effect factor and its receptor during B-cell activation

1213 Pang N. Shek and Albert H. Coons. Effect of colchicine on the antibody response. I. Enhancement of antibody formation in mice

1228 Pang N. Shek, Carl Waltenbaugh, and Albert H. Coons. Effect of colchicine on the antibody response. II. Demonstration of the inactivation of suppressor cell activities by colchicine

1236 T. J. Braciale and K. L. Yap. Role of viral infectivity in the induction of influenza virus-specific cytotoxic T cells

1253 Ben A. M. van der Zeijst, Carleton C. Stewart, and Sondra Schlesinger. Proliferative capacity of mouse peritoneal macrophages in vitro

1267 B. J. Mathieson, P. S. Campbell, M. Potter, and R. Asofsky. Expression of Ly 1, Ly 2, Thy 1, and TL differentiation antigens on mouse T-cell tumors

BRIEF DEFINITIVE REPORTS

1280 Jwu-Sheng Tung, Paul V. O'Donnell, Erwin Fleissner, and Edward A. Boyse. Relationships of gp70 of MuLV envelopes to gp70 components of mouse lymphocyte plasma membranes

1285 Jean-Dominique Vassalli, Angela Granelli-Piperno, Claude Griscelli, and E. Reich. Specific protease deficiency in polymorphonuclear leukocytes of Chédiak-Higashi syndrome and beige mice

1291 Harald von Boehmer, Werner Haas, and Helmut Pohlit. Cytotoxic T cells recognize male antigen and H-2 as distinct entities

Volume 147 No. 5 May 1, 1978

1299 Giorgio Trinchieri, Daniela Santoli, Robert R. Dee, and Barbara B. Knowles. Anti-viral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Identification of the anti-viral activity as interferon and characterization of the human effector lymphocyte subpopulation

1314 Giorgio Trinchieri and Daniela Santoli. Anti-viral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Enhancement of human natural killer cell activity by interferon and antagonistic inhibition of susceptibility of target cells to lysis

1334 Joyce M. Zarling and Fritz H. Bach. Sensitization of lymphocytes against pooled allogeneic cells. I. Generation of cytotoxicity against autologous human lymphoblastoid cell lines

1341 U. Krawinkel, M. Cramer, I. Melchers, T. Imanishi-Kari, and K. Rajewsky. Isolated hapten-binding receptors of sensitized lymphocytes. III. Evidence for idiotypic restriction of T-cell receptors

1348 Lilly Y. W. Bourguignon, Robert Hyman, Ian Trowbridge, and S. J. Singer. Localization of T25 glycoprotein in wild-type and Thy 1^- mutant cells by immunofluorescence and immunoelectron microscopy

1355 P. R. Narayanan and G. Sundharadas. Differential effects of polyadenylic: polyuridylic acid and lipopolysaccharide on the generation of cytotoxic T lymphocytes

1363 Thomas G. Gipson, Masahiro Imamura, McGregor A. Conliffe, and W. John

Martin. Lung tumor-associated derepressed alloantigen coded for by the *K* region of the *H-2* major histocompatibility complex

1374 I. Zan-Bar, E. S. Vitetta, F. Assisi, and S. Strober. The relationship between surface immunoglobulin isotype and immune function of murine B lymphocytes. III. Expression of a single predominant isotype on primed and unprimed B cells

1395 Peter J. Wettstein, Donald W. Bailey, Larry E. Mobraaten, Jan Klein, and Jeffrey A. Frelinger. T-lymphocyte response to *H-2*mutants. I. Proliferation is dependent on Ly 1^+2^+ cells

1405 Carlo E. Grossi, Susan R. Webb, Antonio Zicca, Peter M. Lydyard, Lorenzo Moretta, M. Cristina Mingari, and Max D. Cooper. Morphological and histochemical analyses of two human T-cell subpopulations bearing receptors for IgM or IgG

1418 Arthur K. Kimura and Hans Wigzell. Cell surface glycoproteins of murine cytotoxic T lymphocytes. I. T 145, a new cell surface glycoprotein selectively expressed on Ly 1^-2^+ cytotoxic T lymphocytes

1435 U. Botzenhardt, J. Klein, and M. Ziff. Primary in vitro cell-mediated lympholysis reaction of NZB mice against unmodified targets syngeneic at the major histocompatibility complex

1449 Paul S. Russell, Catharine M. Chase, Robert B. Colvin, and Janet M. D. Plate. Kidney transplants in mice. An analysis of the immune status of mice bearing long-term, H-2 incompatible transplants

1469 Paul S. Russell, Catharine M. Chase, Robert B. Colvin, and Janet M. D. Plate. Induce immune destruction of long-surviving, H-2 incompatible kidney transplants in mice

1487 E. S. Raveché, A. D. Steinberg, L. W. Klassen, and J. H. Tjio. Genetic studies in NZB mice. I. Spontaneous autoantibody production

1503 Phyllis Bodel. Spontaneous pyrogen production by mouse histiocytic and myelomonocytic tumor cell lines in vitro

1517 David S. Pisetsky and David H. Sachs. Genetic control of the immune response to staphylococcal nuclease. VIII. Mapping of genes for antibodies to different antigenic regions of nuclease

BRIEF DEFINITIVE REPORT

1526 David E. Harrison, Clinton M. Astle, and Joan A. Delaittre. Loss of proliferative capacity in immunohemopoietic stem cells caused by serial transplantation rather than aging

Volume 147 No. 6 June 1, 1978

1535 Tova Francus, B. Dharmgrongartama, Richard Campbell, Matthew D. Scharff, and Barbara K. Birshtein. IgG$_{2a}$-producing variants of an IgG$_{2b}$-producing mouse myeloma cell line

1551 Ian C. M. MacLennan and Pierre Golstein. Requirement for hexose, unrelated to energy provision, in T-cell-mediated cytolysis at the lethal hit stage

1568 J. R. Roubinian, N. Talal, J. S. Greenspan, J. R. Goodman, and P. K. Siiteri. Effect of castration and sex hormone treatment on survival, anti-nucleic acid antibodies, and glomerulonephritis in NZB/NZW F$_1$ mice

1584 Eva Engvall, Erkki Ruoslahti, and Edward J. Miller. Affinity of fibronectin to collagens of different genetic types and to fibrinogen

1596 Philippa Marrack and John W. Kappler. The role of H-2-linked genes in helper T-cell function. III. Expression of immune response genes for trinitrophenyl conjugates of poly-L(Tyr, Glu)-poly-D,L-Ala-poly-L-Lys in B cells and macrophages

1611 Alfred Singer, Carol Cowing, Karen S. Hathcock, Howard B. Dickler, and Richard J. Hodes. Cellular and genetic control of antibody responses in vitro. III. Immune response gene regulation of accessory cell function

1621 Mikael Jondal and Stephan Targan. In vitro induction of cytotoxic effector cells with spontaneous killer cell specificity

ever, long-term BM engraftment occurred in only 53% of recipients given 10 × 10^6 cells, and the vigor of GVHD in this strain combination was not tested in recipients prepared with lethal WBI.

The present investigation shows that increasing the dose of injected BM cells from 10 × 10^6 to 30 × 10^6 increased the percentage of recipients with long-term (>100 days) BM grafts from 53 to at least 90%. The percentage of donor-type lymphocytes in the blood also increased from 44 to 91%. All of the chimeric animals maintained skin allografts for more than 100 days, and showed no clinical signs of GVHD (hunched back, weight loss, diarrhea, etc.).

Reduction of the cumulative dose of TLI to 1,400 rads substantially reduced the nonspecific immunosuppressive effects observed with 3,400 rads, since skin allograft survival with the low dose (mean, 18.4 days) was considerably less than that with the high dose (mean, 49.1 days). Reduction of the dose of TLI also decreased the frequency of recipients with long-term BM engraftment to <10% after the injection of 30 × 10^6 BM cells.

The critical importance of irradiation of the thymus during TLI was demonstrated by the marked decrease in the survival of C57BL/Ka skin grafts transplanted to recipients given TLI with thymic shielding as compared to that in recipients given TLI with the thymus exposed. However, fractionated, high-dose irradiation (3,400 rads total, 200 rads/fraction) of the thymus alone resulted in only marginal prolongation of skin graft survival (mean survival, 18 days). None of the recipients given thymic irradiation alone accepted long-term BM grafts. These findings suggest that irradiation of both the thymus and peripheral lymphoid tissues is necessary to achieve the full immunosuppressive effects of TLI.

To study the vigor of the GVHD in the C57BL/Ka → BALB/c strain combination, BALB/c mice were given a single lethal dose (1,000 rads) of WBI and 30 × 10^6 C57BL/Ka BM cells. The majority of recipients died within 12 days, and 95% died within 61 days. All irradiated recipients given 30 × 10^6 syngeneic BM cells survived longer than 250 days. Allogeneic marrow recipients showed typical signs of GVHD with alopecia, weight loss, hunched back, and diarrhea. In contrast, >80% of mice given TLI alone, or TLI and 30 × 10^6 allogeneic BM cells survived at least 110 days after BM transplantation. Although the marrow recipients were chimeric, none showed clinical signs of GVHD. The cause of death of those animals which died in these latter groups is not clear. Autopsy results were similar to those observed in untreated control mice which succumbed in our holding rooms during the same period of time. These findings show that preparation of BM recipients with TLI allows for engraftment without GVHD in a strain combination which shows severe GVHD when WBI is used for preparation.

Although the mechanism by which TLI protects against GVHD remains to be elucidated, the severe GVHD induced by 30 × 10^6 allogeneic spleen cells in recipients given TLI suggests that the usual target organs are susceptible to immunological attack by mature immunocompetent T cells. Protection against GVHD by allogeneic BM cells may be due in part to the lack of development of mature T cells and/or to the development of suppressor cells from immature cells in the BM. Even susceptibility to GVHD induced by spleen cells was transient in mice given TLI, since no GVHD was observed after multiple injections of spleen cells into stable chimeras or into mice allowed to spontaneously recover from TLI for a period of >100 days. It is likely that the allogeneic spleen cells can only proliferate rapidly in the lymphoid tissues before repopulation by either progeny of the allogeneic BM cells, or that of the host's own BM cells.

One important problem in clinical BM transplantation is presensitization by blood transfusions (1). To investigate this point in our experimental model, several BALB/c mice were given 0.25-ml blood transfusions from C57BL/Ka donors on two separate occasions before the initiation of radiotherapy and BM transplantation. The experimental results show that the blood transfusions prevented long-term engraftment of BM in recipients given TLI. This suggests that TLI cannot abrogate immunity to allogeneic BM cells induced by the blood elements. Further studies on the effects of TLI on the rejection of skin allografts in presensitized mice showed that TLI substantially prolongs allograft survival, even in mice that had previously rejected a skin allograft. However, the survival time (mean, 23 days) was less than half of that in unsensitized mice given TLI (mean, 49.1 days).

In conclusion, this work suggests that TLI may be a useful procedure in clinical BM and organ transplantation. TLI has already been used extensively in humans for treatment of lymphoid malignancies, and is a relatively safe form of radiotherapy (10).

Summary

Bone marrow (BM) and skin allografts from C57BL/Ka ($H\text{-}2^{b/b}$) mice were transplanted to BALB/c ($H\text{-}2^{d/d}$) recipients treated with total lymphoid irradiation (TLI), whole-body irradiation (WBI), or fractionated thymic irradiation. TLI prolonged skin allograft survival about five times as long as that in untreated controls, and allowed for permanent engraftment of BM cells in ≅90% of recipients. None of the BM recipients showed clinical signs of graft-versus-host disease (GVHD) (diarrhea, weight loss, hunched back, etc.). On the other hand, recipients given WBI and allogeneic BM cells developed severe clinical GVHD. The majority of the latter recipients died within 12 days after BM transplantation, and 95% died within 61 days. Although TLI protected BALB/c mice against GVHD induced by BM cells, all recipients given TLI and allogeneic spleen cells developed lethal GVHD. Thymic irradiation alone marginally prolonged skin allograft survival, and did not allow for allogeneic BM engraftment. These results suggest that TLI may be a useful regimen in clinical BM transplantation, since this form of radiotherapy is used extensively in humans and has few severe side effects.

We thank G. Garrelts, C. M. Johnson, and D. L. Lee for their technical assistance, and M. Janisch for her secretarial assistance.

Received for publication 5 December 1977.

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ROLE OF COMPLEMENT IN THE PATHOGENESIS OF EXPERIMENTAL AUTOIMMUNE MYASTHENIA GRAVIS*

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Myasthenia gravis (MG)[1] is an organ-specific autoimmune disease of skeletal muscle. Both cellular and humoral immunity to nicotinic acetylcholine receptors (AChR) are demonstrable in patients with MG (1). Experimental autoimmune myasthenia gravis (EAMG) can be induced in animals by a single immunization with solubilized AChR and complete Freund's adjuvant (CFA). The chronic phase of EAMG in rats has proven an excellent animal model for MG (1). EAMG can be adoptively transferred to normal animals by living immune lymph node cells (2, 3) but an effector role has not been demonstrated for T cells in the pathogenesis of EAMG or MG. Helper T cells participate in the rat's autoantibody response to AChR (2, 4). Considerable evidence indicates that anti-AChR antibodies play a central role in impairing neuromuscular transmission both in EAMG and MG (5).

When *Bordetella pertussis* is used as a supplementary adjuvant, a transient acute phase of weakness occurs in Lewis rats 7-8 days after inoculation with AChR (6). Acute EAMG is characterized histologically by infiltration of motor end plates with mononuclear inflammatory cells which separate nerve terminals from the muscle's postsynaptic membrane (7). That membrane is selectively destroyed by intensely phagocytic cells. Acute EAMG can be passively transferred to normal rats by intravenous injection of the IgG fraction from sera of rats with chronic EAMG (8). Usually within 4 days of the onset of weakness, clinical recovery occurs and inflammatory cells disappear.

It is not known what preclinical events lead to simplification of muscle postsynaptic membranes and loss of AChR in spontaneously occurring MG (9). A human counterpart of acute EAMG has not yet been described but occasional association of polymyositis with MG (10) and occurrence of muscle "lymphor-

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* Supported by grants from the National Institutes of Health (NS 11719 and NS 11323), the Muscular Dystrophy Association, Inc., and the Los Angeles Chapter of the Myasthenia Gravis Foundation.

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[1] *Abbreviations used in this paper:* α-BT, α-bungarotoxin; AChR, nicotinic acetylcholine receptors; ADCC, antibody-dependent cell-mediated cytotoxicity; CoF, cobra venom factor; CFA, complete Freund's adjuvant; EAMG, experimental autoimmune myasthenia gravis; EMG, electromyogram; HEAE, hyperacute experimental autoimmune encephalomyelitis; MEPP, miniature end plate potential; MG, myasthenia gravis.

rhages" in MG may be evidence of a similar inflammatory cellular event in MG.

To investigate the role of complement as a potential mediator of the pathogenicity of anti-AChR antibodies in vivo, rats were depleted of complement by treatment with cobra venom factor before injection of anti-AChR antibodies. Other rats were depleted of complement after immunization with AChR to determine whether expression of the acute phase of EAMG was complement dependent.

Materials and Methods

AChR Preparation and Inoculation. AChR was prepared from the electric organs of *Electrophorus electricus* (11) and *Torpedo Californica* (12) as described previously. Female Lewis rats (obtained from Simonsen Laboratories, Gilroy, Calif.), aged approximately 10 wk, were inoculated on a single occasion in multiple intradermal sites either with AChR (5-10 μg) plus CFA and *B. pertussis* vaccine (2), or with adjuvants only.

Antibody Preparation and Inoculation. Immunoglobulins were prepared by 40% ammonium sulfate precipitation from sera of rats immunized 25-100 days earlier with AChR and adjuvants or adjuvants only (8). 1 ml vol containing 40-60 mg protein was injected into the exposed jugular vein under ether anesthesia.

Cobra Venom Factor Preparation and Inoculation. Cobra Factor (CoF) was prepared from the venom of *Naja naja siamensis*, assayed in vitro for anti-complementary activity as described elsewhere (13), and treated with α-paradibromo acetophenone to inhibit phospholipase A_2.[2] A loading dose of CoF (325 U/Kg) was given i.p. in five divided doses over the first 24 h. For subsequent maintenance of C_3 depression, 65 U/Kg was injected every 48 h.

Clinical and Electrophysiological Assessments. Rats were weighed daily and tested for muscle weakness which was graded on a scale ranging from + for weak cry and grip to +++ for moribund as described previously (6). Electromyograms were performed as described previously, and rats lacking spontaneous decrements of muscle action potential with slow repetitive motor nerve stimulation were retested after i.p. injection of 8 μg curare (14). A consistent decrement ≥10% (fifth response compared with first) was considered significant.

Histology. Frozen sections (8 μm) of forelimb extensor muscles were formalin fixed and stained with hematoxylin and eosin after histochemical reaction with α-naphthyl acetate to locate end plate cholinesterase (7).

Quantitation of Muscle AChR and of AChR Complexed in Situ with Antibody. AChR was extracted with 2% Triton X-100 from washed pelleted membranes of homogenized individual rat carcasses (15). An aliquot of each rat extract was incubated overnight with ^{125}I-α-bungarotoxin (α-BT) to label AChR which was then precipitated by sequential addition of excess rat anti-AChR antibodies and goat anti-rat Ig. AChR concentration was expressed in terms of moles of ^{125}I-α-BT precipitated. The proportion of AChR which was already complexed with antibody when extracted from muscle was determined from the cpm of ^{125}I-α-BT directly precipitated by goat anti-rat Ig without addition of anti-AChR antibodies (15).

Antibody Assays. AChR solubilized from normal rat muscle and labeled with ^{125}I-α-BT provided antigen for testing sera in an indirect immunoprecipitation assay (6, 15).

C_3 Assays. Serum C_3 was measured by the Mancini immunodiffusion technique. Anti-rat C_3 antiserum was raised by immunizing rabbits i.d. with an incomplete Freund's adjuvant emulsion containing repeatedly washed precipitates of boiled zymosan reacted with fresh rat serum. The Ig fraction of pooled rabbit antisera was absorbed with rat Ig, and showed a single precipitin band when reacted with whole normal rat serum and little or no reaction with serum of rats depleted of C_3 with CoF. The precipitin band was identified as C_3 in immunoelectrophoretic analysis.

Results

C_3 Values. All experiments were completed within 5 days of commencing

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TABLE I

Effects of Complement Depletion on the Outcome of Passively Transferred Anti-AChR Antibodies at Low and High Doses

Experiment number	Immunoglobulins injected	Treatment	Incidence of			AChR Extracted from muscle	
			Clinical signs	EMG Decrement	Inflammatory cells	Moles $(\times 10^{-11})$ mean ± SE (n)	% Complexed with antibody mean ± SE
I	Anti-AChR (2×10^{-11} mol)	Saline	3/7	6/7	6/6	2.95 ± 0.09 (6)*	19 ± 0.8
	Anti-AChR (2×10^{-11} mol)	CoF	0/4	0/4	0/4	4.38 ± 0.16 (4)	18 ± 1.0
	Nil	Nil	0/5	–	0/3	4.08 ± 0.21 (5)	0
II	Anti-AChR (20×10^{-11} mol)	Saline	5/5	4/4‡	4/4	2.05 ± 0.21 (5)*	53 ± 3.0
	Anti-AChR (20×10^{-11} mol)	CoF	0/5	0/5	0/5	3.56 ± 0.13 (5)	60 ± 2.0
	Adjuvant	CoF	0/2	0/2	0/2	3.55, 4.25	0
	Nil	Nil	0/2	–	–	3.00, 3.16	0

* Values for AChR extracted from muscle of antibody recipients treated with saline were significantly lower than controls ($P <$ 0.001, Student's t test). Values for antibody recipients treated with CoF did not differ from controls.

‡ One rat died before testing. The four survivors had + +/+ + + weakness and spontaneous decrements of muscle action potential on repetitive motor nerve stimulation; rats in all other groups were tested after challenge with curare, and none showed a decrement except the saline-treated recipients of the lower dose of antibodies.

CoF and the mean serum C_3 value of rats treated with CoF (48 h earlier) was 11% of that in rats treated with saline.

Effects of Passively Transferred Anti-AChR Antibodies. Two separate experiments were performed by employing syngeneic Ig preparations which differed 10-fold in their content of antibodies to muscle AChR (2 and 20×10^{-11} mol per ml, respectively). This allowed comparison of the effects of antibody doses less than and in excess of the total amount of AChR extractable from an average rat's musculature ($3\text{-}5 \times 10^{-11}$ moles). CoF (or saline) was commenced 2 days before Ig injection and rats were killed by ether inhalation 3 days after Ig injection, immediately after electromyogram (EMG) tests.

Rats treated with saline, i.e. having normal C_3 levels, developed clinical and electrophysiological signs of EAMG after injection of anti-AChR antibodies (Table I). The smaller dose of antibodies (2×10^{-11} moles) used in experiment I induced mild clinical signs (+), without a spontaneous EMG decrement, but enhanced curare sensitivity was demonstrated electromyographically. There was a 32% reduction in muscle AChR. In experiment II, rats injected with a larger dose of antibodies (20×10^{-11} moles) had clinically more severe EAMG (+ +/+ + +) with profound weight loss (Fig. 1), and spontaneous EMG decrements; muscle AChR was 41% less than that of control groups which did not receive anti-AChR antibodies. Inflammatory cells, predominantly mononucleated, were found in the motor end plate regions of muscle from recipients of both high and low doses of antibodies. The inflammatory cells were intimately associated with cholinesterase-positive material (i.e. postsynaptic membrane) which, in many instances, was sequestered away from the muscle fiber and in a granular, disintegrated form (Fig. 2).

Injection of anti-AChR antibodies into rats depleted of C_3 by CoF treatment, induced neither weakness, significant weight loss (Fig. 1), a decrementing

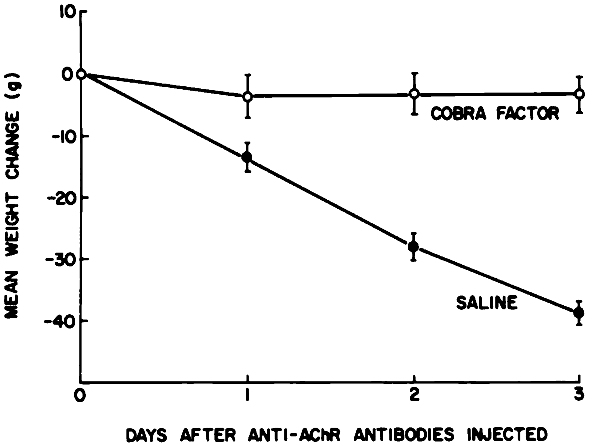

FIG. 1. Mean weight changes (± SD) of two groups of five rats injected on day 0 with 20 × 10^{-11} mol anti-rat muscle AChR antibodies (mean starting weight = 195 ± 12 g). The group treated with CoF had neither clinical signs of weakness, nor enhanced curare sensitivity; the group treated with saline had ++/+++ EAMG and spontaneous decrements of muscle action potential when tested by EMG with repetitive motor nerve stimulation.

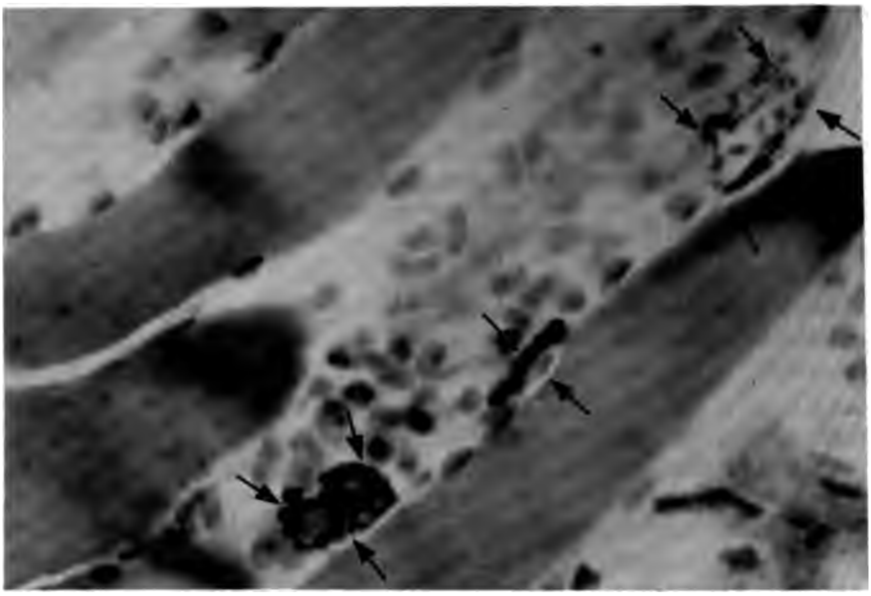

FIG. 2. Motor end plate region of digit extensor muscle from a rat 72 h after being treated with saline and injected i.v. with anti-AChR antibodies. Numerous inflammatory cells surround three areas of darkly stained cholinesterase reaction products (arrows) which are sequestered from muscle fibers. Granular appearance is particularly evident in the area on the right (Fig. 3). (hematoxylin and eosin, × 400).

response to repetitive nerve stimulation, nor an increased curare sensitivity (Table I). The amount of AChR solubilized from muscle did not differ significantly from that of control rats not injected with anti-AChR antibodies. However, AChR extracted from rats treated with CoF was found to have

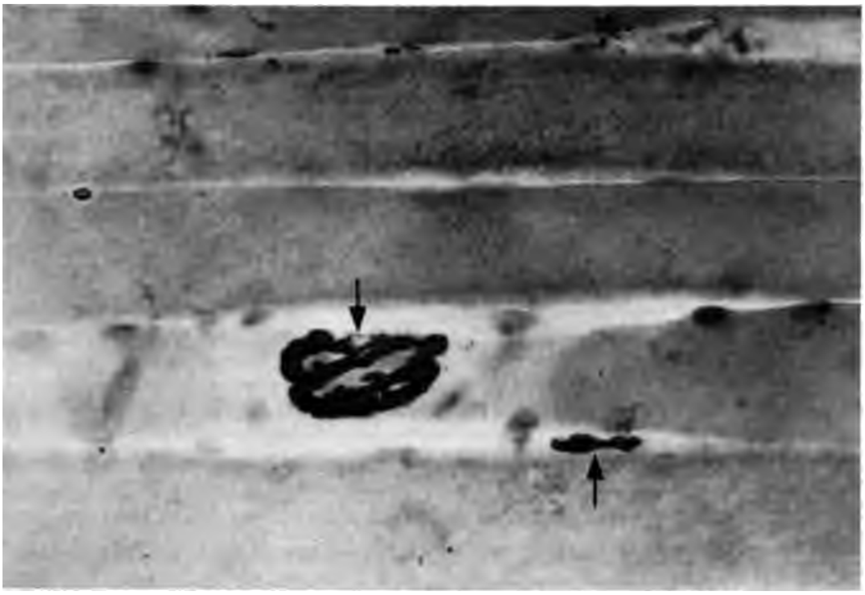

FIG. 3. Motor end plate region of digit extensor muscle from a rat 72 h after being decomplemented with CoF and injected i.v. with anti-AChR antibodies. Two areas of darkly stained cholinesterase reaction products (arrows) are seen from above and from the side. They are compact, have a normal appearance, and can be demonstrated to be closely applied to the muscle fibers. No inflammatory cells are seen. (hematoxylin and eosin, × 400).

antibody bound to it, and, indeed, the absolute amount of AChR complexed with antibody was greatest in C_3-depleted rats injected with the larger dose of antibodies (Table I). Motor end plate regions (Fig. 3) lacked inflammatory cells, and the cholinesterase reaction products resembled those of normal rats and rats injected with adjuvant Ig alone.

Evidence for Complement's Role in Destruction of the Postsynaptic Membrane in the Acute Phase of EAMG Induced by Immunization with AChR. C_3 has been reported to play a role in the induction of immune responses to T-dependent antigens (16). Thus, to ascertain whether our protocol for CoF administration might inhibit induction of T-cell responses in Lewis rats, a preliminary experiment was performed in another model disease – hyperacute experimental autoimmune encephalomyelitis (HEAE). HEAE induced in Lewis rats by guinea pig myelin basic protein has the same time-course as acute EAMG (17) but immunopathologic lesions appear to be mediated entirely by effector T cells. Injection of CoF from the 7th day after inoculation with 30 μg guinea pig myelin basic protein plus CFA and *B. pertussis* caused depletion of serum C_3, but did not delay the onset or inhibit the severity of HEAE.

To investigate whether complement may have an effector role in the acute phase of EAMG induced by active immunization, CoF treatment was commenced on the 7th day after inoculation with Torpedo. AChR. Of 18 rats inoculated with AChR and adjuvants, 6 received CoF, and 12 received saline. When sacrificed on the 11th postinoculation day (the expected time of peak incidence of acute EAMG), serum titers of antibody to Torpedo AChR in the

TABLE II

Effect of CoF on the Acute Phase of EAMG Induced by Active Immunization with Torpedo AChR

Immunogen	Treatment	Incidence of			AChR Extracted from muscle		
		Clinical signs	EMG Decrement	Inflammatory cells	Range (moles × 10^{-11})	% Complexed with antibody	
						Mean	Range
AChR	Saline	8/12	8/11*	7/10	3.2 → 8.5‡	3.2	(1-6)
AChR	CoF	1/6	0/6	0/4	5.1 → 5.4	1.0	(0-2)
Adjuvant	CoF	0/5	0/5	0/3	5.0 → 5.6	0	–

* Six rats had spontaneous decrements of muscle action potential on repetitive motor nerve stimulation, and two more in this group, but none in the other groups, showed a decrement after challenge with curare.

‡ A wide range of values for muscle AChR is characteristic of acute EAMG (see text).

group treated with CoF (mean ± SD = 1.99 ± 0.85 $\times 10^{-7}$ M, $n = 4$) were in the lower range of titers of the group treated with saline (3.65 ± 1.38, $n = 11$). This difference ($P = 0.05$, Wilcoxon's rank test) suggests that injection of CoF slightly depressed the humoral immune response to AChR, which requires participation of helper T cells (2, 4). One rat treated with CoF developed mild weakness on day 9 but none had EMG evidence (curare test) of impaired neuromuscular transmission. 8 of 12 rats treated with saline developed weakness ranging from + to +++ severity, and EMG evidence of impaired neuromuscular transmission (Table II). Three of the eight rats with clinical and electrophysiological signs of acute EAMG had antibody titers in the same range as rats treated with CoF.

The amount of AChR extracted from the musculature of rats inoculated with AChR and treated with CoF (5.1-5.4 $\times 10^{-11}$ mol) did not differ significantly from that of controls inoculated with adjuvants (5.0-5.6 $\times 10^{-11}$ mol), whereas values in rats inoculated with AChR and treated with saline varied over a wide range (3.2-8.5 $\times 10^{-11}$ mol). A wide range of muscle AChR content is characteristic of acute EAMG (15), and presumably reflects variable loss and resynthesis of AChR. In both saline and CoF-treated groups, only a small amount of the extracted AChR had antibody bound to it. This also is characteristic of the acute phase of EAMG in normal rats (15).

In 7 of 10 muscle biopsies available from the saline-treated group, mononuclear cells were abundant and in intimate association with granular cholinesterase-positive material. The cholinesterase-positive material (i.e. postsynaptic membrane) was frequently detached from muscle fibers. The motor end plate regions of four rats examined after CoF treatment (including the one with mild weakness) resembled those of control rats inoculated with adjuvants only: the cholinesterase-positive material was compact and closely applied to muscle fibers with no evidence of inflammatory cells.

Discussion

The present study has established that complement plays a critical role in vivo in mediating impairment of neuromuscular transmission in the acute phase of EAMG induced either by passive transfer of anti-AChR antibodies or by active immunization with AChR. Injection of syngeneic anti-AChR antibodies in excess of the muscle's content of AChR was without any measurable effect in rats treated with CoF. There was no weakness or impairment of neuromuscular transmission as judged electromyographically by curare sensitivity, and the absolute amount of AChR was uniformly within the normal range despite complexing of 60% of AChR with antibody.

Had binding of antibodies to the postsynaptic membrane induced redistribution (4, 12) and accelerated degradation of surface AChR (18–21), as have been reported in vitro from studies with both rat and human anti-AChR antibodies on extrajunctional AChR of cultured muscle cells, one might expect an increase in curare sensitivity and also some alteration (either a decrease, or increase, by 72 h) in the amount of AChR extractable from muscle. Thus if antibody-induced modulation of AChR does occur in the intact adult neuromuscular junction, that mechanism does not appear to contribute significantly to the reduction of ACh sensitivity of the postsynaptic membrane induced in vivo by passive transfer of anti-AChR antibodies.

Impairment of the ionophore function of AChR by antibodies has also been suggested from in vitro studies as a mechanism by which antibodies might impair neuromuscular transmission. Again one might anticipate an increase in curare sensitivity, if this mechanism were a significant pathogenic factor. However, since neuromuscular transmission in the rat has such a large "safety factor", these apparently minor mechanisms of impairing neuromuscular transmission could be of importance in membranes whose content of AChR has been greatly reduced by complement-dependent mechanisms.

Observations by Toyka et al. (22) in mice injected with IgG from patients with MG, suggested that components of the complement system activated early in the cascade were important mediators of neuromuscular transmission impairment by anti-AChR antibodies in vivo. They found that recipient mice treated with CoF developed less severe signs of MG (reduction of miniature end plate potential [MEPP] amplitude and decreased α-BT binding sites in muscle) than did normal recipient mice. In contrast, recipient mice genetically deficient in C_5 were affected as severely by MG immunoglobulins as normals. This implied that lytic components of complement were not involved in impairment of neuromuscular transmission by antibodies and complement.

The present study has provided evidence for a complement-dependent mechanism of membrane destruction which need not require the terminal components of complement. Activation of complement by antibodies binding to AChR in the postsynaptic membrane appears to provide a signal that is directly or indirectly chemotactic for mononuclear inflammatory cells. Inflammatory cells did not accumulate in the motor end plates of rats depleted of complement either before passive transfer of anti-AChR antibodies or 7 days after active immunization with AChR. A destructive mononuclear cellular response, which is similarly both transferable by antibodies and complement-dependent, has been described in a guinea pig model of experimental autoimmunity to renal tubular basement membranes (23). Electron microscopy has revealed that destruction of the postsynaptic membrane in acute EAMG is effected principally by cells resembling macrophages (7). Nonimmune lymphoid cells which attack the postsynaptic membrane presumably bear receptors for C_3 and the Fc piece of IgG complexed with antigen. We previously reported that anti-AChR antibodies detectable in the serum of rats with EAMG (day 10) were predominantly 7S (2). It has been demonstrated in the rabbit that homologous anti-AChR antibodies are cytophilic for macrophages (24). C_3 receptors on lymphoid cells have been proposed to play a role in vivo in promoting antibody-dependent cell-mediated cytotoxicity (ADCC), which is inhibited in vitro by concentrations of IgG which exist in vivo (25). Furthermore, Fc receptors on lymphoid cells have been reported to bind inefficiently to particle bound Fc (16). Thus IgG and C_3 probably act synergistically in opsonization and phagocytosis of particulate antigens. The binding of a macrophage's C_3 receptors to the activated form of C_3 on the postsynaptic membrane would promote interaction between its Fc receptors and IgG on the postsynaptic membrane, thus triggering phagocytosis.

If this picture of acute EAMG is correct, then the absence of inflammatory cells at the motor end plate in chronic EAMG must be explained. First, it is possible that after initial destruction of the postsynaptic membrane by complement-dependent mechanisms, immune complexes (AChR, IgG, and C_3) on the remaining membrane are no longer of sufficient number or in optimal array for effective interaction with lymphoid cells bearing C_3 and Fc receptors, and no longer give rise to an adequate chemotactic signal for inflammatory cells. This hypothesis would most readily explain the abrupt termination of the acute phase of EAMG. Pronounced clinical recovery from acute EAMG, which is often observed over the course of 8 h (V. Lennon, unpublished observation), probably reflects return of the nerve terminals into proximity to remnants of the postsynaptic membrane after disappearance of inflammatory cells. A second possible explanation for termination of acute EAMG is that Fc and C_3 receptors on effector lymphoid cells might become occupied by soluble complexes of antigen and antibody appearing in the circulation with progression of the immune response induced by inoculation with AChR and adjuvants. Because the ratio of antigen and antibody is critical for effecting ADCC in vitro (26), a third possibility is that the transient increase in muscle AChR which follows separation of nerve terminals from postsynaptic membranes by inflammatory cells (15), might inhibit lymphoid cells from binding to postsynaptic immune complexes. In MG, a possible explanation for rarity of inflammatory cells at neuromuscular junctions could be that the Fc piece of the IgG subclass(es) of human anti-AChR antibodies may not be cytophilic for lymphoid cells.

Deposition of homologous C_3 has been demonstrated by indirect immunofluorescence on the membranes of cultured muscle cells exposed to anti-AChR antibodies from rats with acute or chronic EAMG (4). Although cultured myotubes were not lysed after binding homologous antibody and complement (4), complement-mediated lysis may occur in vivo in the highly specialized postsynaptic membrane. Engel and co-workers have demonstrated focal lysis of the tips of postsynaptic membrane folds electron microscopically in MG (27), and in chronic EAMG, and also early in acute EAMG in the absence of inflammatory cells (7). Immunoelectron microscopic studies of the neuromuscular junction in both chronic EAMG (28) and MG (27) suggest that lysis of the postsynaptic membrane is complement-mediated—both IgG and C_3 were demonstrated on degenerating membrane fragments in the synaptic cleft. C_3 was demonstrated also on the postsynaptic membrane in the same distribution as bound IgG (27) and residual AChR (i.e. α-BT binding sites) (9). In mild cases of MG, where ultrastructural abnormality of the postsynaptic membrane and reduction of AChR were minimal, a maximal area of membrane was covered by IgG and C_3, but reduction in MEPP amplitude was at most only 40% (27). In more clinically severe cases of MG, MEPP amplitude reduction was paralleled by reduction in AChR, IgG, and C_3 on the postsynaptic membrane. It could be concluded from those studies that in severe cases of MG the mere presence of IgG and C_3 on the postsynaptic membrane was not the major cause of the marked reduction in MEPP amplitude; membrane destruction and accompanying loss of AChR (29) were more important.

The present study in decomplemented rats has provided direct evidence that binding of antibody to AChR at the neuromuscular junction does not significantly compromise neuromuscular transmission unless the amount of AChR also is reduced. Complement appears to play a central role in vivo, at least in the acute phase of EAMG in rats, in mediating both destruction of the postsynaptic membrane and loss of AChR which is initiated by the binding of anti-AChR antibodies.

Summary

An acute phase of experimental autoimmune myasthenia gravis (EAMG) occurs transiently early in the immune response of Lewis rats to nicotinic acetylcholine receptors (AChR) when *Bordetella pertussis* is used as adjuvant. It is characterized by a destructive cellular attack directed at the postsynaptic membrane of muscle. Acute EAMG can be passively transferred to normal rats by IgG from serum of rats with chronic EAMG. In the present study, acute EAMG, induced either by passive transfer of syngeneic antibodies or by active immunization, was inhibited in rats depleted of complement by treatment with cobra venom factor (CoF). Furthermore, passive transfer of antibodies in excess of the muscle's content of AChR was without any measurable effect in rats treated with CoF. Although 60% of the muscle's AChR was complexed with antibody, there was no reduction in the muscle's content of AChR, and neuromuscular transmission was not compromised as judged electromyographically by curare sensitivity. These data imply that redistribution, accelerated degradation, and impairment of the ionophore function of AChR, effects of antibodies described in vitro on extrajunctional AChR, do not play a significant role in vivo in impairing neuromuscular transmission in an intact neuromuscular junction. Complement appears to be a critical mediator of anti-AChR antibodies' pathogenicity in vivo.

We thank Millie Thompson, Ruth Rubin, Brett Einarson, and Beki Baergen for excellent technical assistance, and Eli Lilly and Co., Indianapolis, Ind. and Sterling Drug Inc., Rensselaer, N. Y., for gifts of *B. pertussis* and benzoquinonium.

Received for publication 5 December 1977.

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EXPRESSION OF IgD BY MURINE LYMPHOCYTES

Loss of Surface IgD Indicates Maturation of Memory B Cells*

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Antibody-forming cell (AFC)[1] precursors arise from cells in the bone marrow and differentiate to antigen-specific B cells. On exposure to antigen, in the presence of T lymphocytes and macrophages, these B cells further differentiate to effector cells – AFC that secrete large amounts of immunoglobulin (Ig) with a specificity for antigen identical to that of the receptor on the stimulated cell, or to memory B cells – lymphocytes capable of rapid expansion and differentiation to effector cells upon re-exposure to antigen.

Antibody genes in mammalian cells comprise at least three families, one encoding for κ-light chains, another for λ-light chains, and a tightly linked cluster of genes encoding for heavy chains (1). The surface Ig on a single B cell is homogeneous with respect to the light chain family expressed and to the specificity for antigen (2, 3). Considerable evidence has now accumulated showing that Igs with two or more different Ig heavy chain (Ig-H) classes are present on the surface of a single B cell (4). The expression of different heavy (H) chains and combinations of H chains on B cells has been studied in an effort to chart the ontogeny of, and to distinguish between, functionally different B cells.

In newborn mice most B cells carry only surface IgM. From 4 to 6 wk after birth the majority of B cells in the immune system have both surface IgM and IgD. The developmental relationship between these two cell populations has not yet been established (5). Studies with anti-IgM treatment of newborns have shown that IgM-bearing cells are precursors of IgG and IgA-producing cells (6); however, these studies have not established whether the precursors are IgM only or IgM-IgD-bearing B cells.

In the adult mouse, several investigations have been conducted to establish which Ig classes (isotypes) are found on B cells responding in primary responses and which are found on B cells responding in secondary responses. In T-independent primary responses, both the IgM-only and IgM-IgD B-cell populations were shown to give rise to AFC (7). In T-dependent primary responses, Coffman and Cohn (7) found that only the IgM-IgD B

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* Supported, in part, by grants from the National Institute of General Medical Sciences (GM 17367), National Cancer Institute (CA-04681), and the National Institute of Child Health and Human Development (HD-01287).

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[1] *Abbreviations used in this paper:* AFC, antibody-forming cells; BRBC, burro erythrocytes; DNP-KLH, 2,4-dinitrophenyl conjugates of keyhole limpet hemocyanin; F*, conjugated with fluorescein, fluoresceinated; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; Ig^a, Ig with Ig-1a linked allotypes; Ig^b, Ig with Ig-1b linked allotypes; Ig-5, locus for IgD heavy chain, δ; Ig-6, locus for IgM heavy chain, μ; Ig-H, Ig heavy chain; i.v., intravenous (via a lateral tail vein); MEM, minimum essential medium; PAGE, polyacrylamide gel electrophoresis; PFC, plaque-forming cells; SDS, sodium dodecyl sulfate.

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cells responded; however, Zan-bar et al. (8) also obtained a small T-dependent response with IgD^- (IgM only) B cells as well.

In secondary responses, most of the responding B cells (memory cells) carry IgG (7-11). Some of these IgG-bearing cells carry IgD as well (7). There are also apparently some IgG memory cells that carry little or no surface IgG (9, 10). Thus, the appearance of surface IgG on a B cell defines a major B-cell maturation step; however, the information presently available on expression of surface IgD is inadequate to fit the presence or absence of this Ig isotype into a scheme of B-cell maturation.

In this publication, we present data from a comprehensive study of the presence of IgD on B cells at different stages of maturation, from unprimed lymphocytes to AFC. We show that (*a*) T-dependent primary antibody responses are generated mostly, if not entirely, by IgD-bearing B cells; (*b*) early IgG memory cells carry surface IgD; (*c*) mature (later, higher avidity) IgG memory cells do not carry surface IgD; and (*d*) most IgM AFC carry IgD whereas few (if any) IgG AFC carry IgD. Thus evidence from this study indicates that, with respect to T-dependent-responding B cells, the presence of surface IgD distinguishes less mature from more mature B cells, and can, therefore, be used in conjunction with evidence from other studies to construct an outline for a B-cell maturation pathway.

Materials and Methods

Experimental Approach. Alloantisera with specificity for mouse IgD were used to identify splenic B cells with surface IgD (12). The reaction of such sera with their target cells was visualized by indirect immunofluorescence microscopy and analysis on the fluorescence-activated cell sorter (FACS) (13). IgD^+ (stained) and IgD^- (unstained) populations in the suspension were then isolated with the FACS and tested for functional activity in an adoptive transfer assay. The isolated populations were supplemented with an appropriate source of T cells in these assays to allow full expression of B-cell activity. Three functionally distinct populations of cells involved in antibody responses were examined for expression of surface IgD: unprimed B cells, primed (memory) B cells, and IgM and IgG plaque-forming cells (PFC).

Mice. Two pairs of Ig-H chain congenic mouse strains were used for this work: SJL/JHz, which carries the Ig^b haplotype, and SJA/9Hz, which carries the Ig^a haplotype derived from BALB/c; and BALB/cNHz (Ig^a haplotype) and BAB/14Hz, which carries the Ig^b haplotype of C57BL/Ka (14). Male or female mice between 3 and 6 mo of age were used in these experiments.

Antisera. Mouse anti-Ig-5b (anti-δ of the *b* allotype) was raised by injecting SJA female mice (Ig^a) with 10^7 washed spleen cells from female BAB/14 mice (Ig^b). The BAB/14 spleen cells were administered i.p. on five occasions each separated by 1 wk. Recipient mice were bled 7 days after the last injection of antigen and serum pools were made on the basis of anti-Ig-5b titer. Serum producers were bled weekly, and boosted monthly, and no drop in titer was observed over a $2^1/_2$ mo period. After $2^1/_2$ mo, the serum producers generate a large amount of antibodies that react with antibody complexes. Anti-IgD titer was assessed after absorption of the sera with a Ig-6b myeloma (C.BPC112)-Sepharose or C57BL/6 globulins-Sepharose conjugates. This procedure removes anti-Ig-6b activity from the serum in addition to any antibodies that react with complexed Ig. The anti-Ig-5b activity in absorbed sera was assessed by staining of SJL spleen cells and analysis on the FACS. This procedure permits an objective assessment of how brightly a given serum in combination with a fixed amount of fluoresceinated second step reagent will stain a fixed number of spleen cells. Serum collected over the $2^1/_2$-mo period after the initial immunizations was used in the following studies.

The specificity of the anti-Ig-5b serum has been characterized in two ways: (*a*) By immunofluorescence analysis. These data have been published elsewhere (12). To the already published results we can add that our anti-Ig-5b sera do not stain any cells in SJL fetal liver nor is any activity removed from these sera by absorption with SJL fetal liver cells. These sera also do not stain any cells in the 1-day old SJL spleen whereas 5-10% of these cells can be stained with a rabbit or goat-anti-IgM serum or with anti-Ig-6b serum. In the 4-day old SJL spleen about 5% of cells can be stained with our anti-Ig-5b serum whereas about 20% of these spleen cells are IgM bearing. In the adult spleen, most cells which have membrane IgM also stain for surface Ig-5 (IgD) (5, 7).

(*b*) By SDS polyacrylamide gel (SDS-PAGE) analysis of immunoprecipitates prepared from lysates of radiolabeled SJL spleen cells (S. Black, unpublished data). Briefly, anti-Ig-5b serum that did not react with IgM as shown by radioimmune analysis[2] or staining of 1-day old mouse spleen cells, precipitated only IgD from radiolabeled SJL spleen cell lysates. IgD was identified by the migration pattern in 10% SDS-PAGE under reducing conditions, as a molecule that had a mol wt of approximately 65,000 daltons.

Fluorescein Conjugated Ig^b anti-Ig^a Allotype Serum. This serum was raised by injecting SJL mice with complexes of *Bordetella pertussis* anti-*B. pertussis* antibodies from BALB/cN mice. Ouchterlony analysis showed reaction against Ig-1a, Ig-3a, and Ig-4a. The conjugation of this serum with fluorescein followed the protocol of Cebra and Goldstein (15). Fluoresceinated antiserum was then absorbed with SJL spleen cells until it reacted only with mouse B cells bearing Ig-1a, Ig-3a, or Ig-4a.

Antigens and Priming. Burro erythrocytes (BRBC) in Alsever's solution (Colorado Serum Co., Denver, Colo.) were washed three times in minimum essential medium (MEM) and 5×10^7 were injected i.p. into experimental animals. Keyhole limpet hemocyanin (KLH) (Pacific Bio-Marine Laboratories, Inc., Venice, Calif.) and 2,4-dinitrophenyl (DNP) conjugates of KLH were prepared as previously described (16). Donors were primed with 100 μg DNP-KLH on alum (hapten priming) or with 100 μg KLH on alum (carrier priming). Both antigens were injected i.p. with 2×10^9 killed *B. pertussis* (kindly supplied by the Commonwealth of Massachusetts, Department of Public Health, Boston, Mass.) Mice were used as donors a minimum of 6 wk after priming.

Preparation of B Cells, T Cells, and Erythrocyte-Depleted Spleen Cells. (*a*) T-cell killing: T cells were depleted from spleen cell suspensions by killing with anti-Thy-1 plus complement in a two-step killing protocol. Cells were incubated with congenic anti-Thy-1.2 (kindly supplied by Doctors E. A. Boyse and F. W. Shen, Sloan-Kettering Institute, New York) for 30 min at 37°C. After the first step, cells were pelleted through fetal calf serum (FCS), then resuspended and incubated with agarose-adsorbed guinea pig complement for 30 min at 37°C. After the second step, cells were resuspended and washed once before transfer. (*b*) T-cell-enriched populations of splenocytes were prepared by passing spleen cells through nylon wool columns (17). (*c*) Erythrocyte-depleted splenocytes: erythrocytes were lysed by incubating the cells for 2 min at 4°C in Gey's balanced salt solution in which the NaCl was replaced with an equimolar concentration of NH_4Cl (hemolytic Gey's).

Immunofluorescent Staining for Surface IgD. IgD was visualized on B-cell surfaces by indirect immunofluorescence (12). Splenic lymphocytes harvested from SJL mice in MEM with 5% FCS were stained with antiserum specific for Ig-5b for 15 min at 4°C and pelleted through FCS. The cell pellet was resuspended in fluoresceinated (F*) *b* anti-*a* allotype serum (fluorescein-labeled second step reagent) and incubated for a further 15 min at 4°C. This suspension was again pelleted through FCS and resuspended to a concentration of 10^7 cells/ml for separation by using the FACS-II. Concentrations of the first and second step reagents were chosen to give optimal staining. Visual analysis of stained cells on a ultraviolet Zeiss microscope, (Carl Zeiss, Inc., New York) showed that 30–40% of the population was fluorescent. No cells were stained by the second step reagent alone or after incubation with normal mouse serum.

Sorting and Analysis with FACS-II. FACS isolation used in these experiments allows separation of cells according to amount of bound fluorescein, amount of low-angle light scattering (size), or a selected combination of these two parameters. In these studies scatter gates or thresholds were set so that only small, live lymphocytes were analyzed or separated (18).

Adoptive Transfer and Plaque-Forming Cell Assays. Spleen cells (from SJL mice) in MEM were mixed with T cells as desired just before i.v. injection into BALB/c recipients, X-irradiated (650 rads) 18 h previously. Recipients were challenged at time of transfer with 10 μg aqueous DNP-KLH or 5×10^7 washed BRBC and killed 7 days later. Direct DNP or BRBC PFC in recipient spleens were scored by the method of Cunningham and Szenberg (19). Indirect splenic PFC were counted as the increase in DNP or BRBC PFC in chambers containing an optimal concentration

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[2] S. J. Black, J. W. Goding, G. A. Gutman, L. A. Herzenberg, M. R. Loken, B. A. Osborne, W. van der Loo, and N. L. Warner. Manuscript in preparation.

TABLE I

The Primary Adoptive Anti-BRBC Response

B Cells*	T Cells‡	BRBC	Anti-BRBC PFC $(\times 10^3)$/spleen	
			Direct	Developed
10^7	--	+	1.0	0.9
–	10^7	+	<0.1	<0.1
10^7	10^7	+	14.2	25.1
10^7	10^7 α-Thy-1.2 + C′	+	1.1	3.0
10^7	10^7	–	0.5	0.6

Mixtures of SJL B and T cells were injected into X-irradiated (650 rads) BALB/c mice together with 5×10^7 BRBC. Recipient mice were killed 7 days later and spleen cells were harvested and plaqued against BRBC. The data is tabulated as anti-BRBC PFC $(\times 10^3)$/spleen. Direct PFC are mainly IgM-secreting PFC; developed PFC are mainly IgG-secreting PFC.

* B cells prepared by treating unprimed SJL spleen cells with anti-Thy-1.2 and complement.

‡ Nylon wool-enriched populations.

of facilitating antiserum (rabbit anti-mouse IgG sera for IgG PFC development [10]) over the response in chambers with no facilitating antiserum. Results are expressed as PFC $(\times 10^3)$/spleen.

Because we have used allogeneic recipients for most of the work described here we present the data in Table I to show that the allogeneic adoptive primary anti-BRBC response has similar characteristics to syngeneic T-dependent adoptive primary responses. B cells or T cells alone generate virtually no anti-BRBC PFC in the presence of antigen. Mixtures of B and T cells, when stimulated with BRBC, give rise to substantial numbers of IgM and IgG PFC. Without antigen, few PFC were produced. T cells treated with anti-Thy-1.2 plus complement before mixing with B cells and antigen do not support the response. Thus, although SJL spleen cells were responding to BRBC in an allogeneic environment, the response was T-dependent and required antigen. The immune response obtained was threefold higher than that arising in a syngeneic transfer making this adoptive transfer system attractive for assaying the low responses usually obtained in adoptive primary studies.

Adoptive secondary anti-DNP responses in allogeneic environments also had the characteristics of a syngeneic response (see Table II). DNP-primed B cells together with carrier-primed spleen cells and DNP-KLH generated a strong IgG anti-DNP PFC response but no IgM PFC. Neither DNP-primed B cells nor carrier-primed T cells alone, or carrier-primed T cells and unprimed B cells (with antigen) generated an immune response on challenge with DNP-KLH. Further, after anti-Thy-1.2 plus complement treatment of the carrier-primed cells (Table II, line 4) DNP-primed B cells gave no adoptive anti-DNP-PFC response. Antigen given to the adoptive recipient was always required for a response in the allogeneic environment. This secondary allogeneic transfer gave rise to two to five times more PFC than were obtained in the syngeneic comparison with no changes in cell or antigen requirements (S. Black, unpublished data).

Avidity of DNP-PFC. Avidity of DNP-PFC was estimated by including ε-DNP-lysine in the plaquing chambers at 10-fold concentration steps from 10^{-10} to 10^{-5} M as previously described (20). The number of cells placed into each Cunningham chamber was adjusted so that in the absence of any inhibitor, 250 PFC/chamber were counted. Six replicate chambers were counted for each concentration of inhibitor. For concentrations of ε-DNP-lysine, less than 10^{-6} M the standard error obtained on each set of six replicate PFC counts was $< \pm 7\%$.

Results

Staining of SJL Spleen Cells with Anti-Ig-5b Serum and Separation of IgD$^+$ and IgD$^-$ Lymphocytes. An SJL spleen cell preparation was indirectly stained

TABLE II

Secondary Adoptive Anti-DNP Response

DNP-Primed* B cells	KLH-Primed‡ spleen	DNP-KLH	Anti-DNP PFC (×10^3)/spleen	
			Direct	Developed
5 × 10^6	10^7	+	<0.1	102.5
5 × 10^6	--	+	<0.1	11.5
--	10^7	+	<0.1	8.5
5 × 10^6	10^7 α-Thy-1.2 + C′	+	<0.1	10.1
5 × 10^6	10^7	−	<0.1	3.5

Mixtures of DNP-primed B cells and KLH-primed SJL spleen cells were injected into X-irradiated (650 rads) BALB/c mice together with 10 μg aqueous DNP-KLH. Recipient mice were killed 7 days later and spleen cells were harvested and plaqued against DNP-BRBC. The data are tabulated as anti-DNP PFC (×10^3)/spleen.

* DNP-primed splenic B cells were obtained from SJL mice primed 6 wk previously with DNP-KLH. Whole spleen cell preparations from these mice were treated with anti-Thy-1.2 plus complement.

‡ These cells were harvested from SJL mice primed 6 wk previously with KLH.

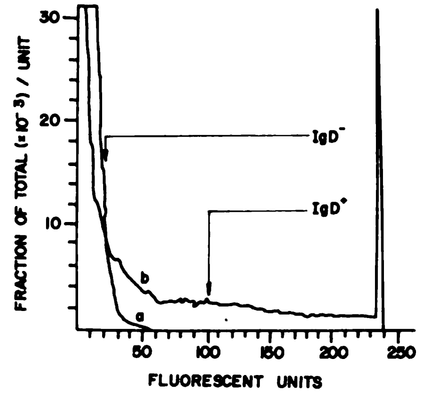

FIG. 1. FACS Analysis of SJL spleen cells stained indirectly with anti-Ig-5b serum. a) Viable small SJL spleen cells stained indirectly with SJA normal mouse serum followed by F*b-anti-a allotype serum. b) Viable small SJL spleen cells stained indirectly with SJA anti-Ig-5b serum followed by F*b-anti-a allotype serum. Separation gates used to isolate IgD^+ and IgD^- lymphocytes are shown. 32% of the anti-Ig-5b stained spleen cell preparation fell between 0 and 20 fluorescence U and was taken as IgD^-. 28% of the anti-Ig-5b stained spleen cell preparation fell between 100 and 240 fluorescence U and was taken as IgD^+.

with specific *a* anti-Ig-5b serum in combination with F**b* anti-*a* allotype serum. UV Zeiss microscope analysis of this cell suspension showed that about 30% of the population was fluorescent. The stained cells were analyzed on the FACS-II. Separation thresholds were set as follows (see Fig. 1): unstained (IgD^-) cells were taken to be those that fell into the first one or two channels. These cells could not be distinguished from low autofluorescent cells. IgD^+ cells were taken as those brightly staining cells that were clearly separate from the "tail" of the IgD^- population (see Fig. 1). Cells that fell between brightly fluorescent (IgD^+)

TABLE III

Expression of IgD on the Primary Responsive B Lymphocyte

B Cells (5×10^6)	T Cells	Anti-BRBC PFC ($\times 10^3$)/spleen	
		Direct	Developed
Stained unseparated spleen	10^7	14	25
IgD^+ fraction	10^7	13	22
IgD^- fraction	10^7	<0.1	<0.1
--	10^7	<0.1	<0.1

SJL spleen cells were stained with SJA anti-Ig-5b serum and F*b anti-a allotype serum, then sorted into bright IgD^+ (top 28%) and dull IgD^- (bottom 32%) fractions. Recipients were X-irradiated (650 rads) BALB/c mice. Washed BRBC (5×10^7) were injected along with lymphocytes. T cells are nylon wool-enriched spleen cells. The PFC response was assayed 7 days after transfer.

and dull fluorescent (IgD^-) were discarded. Details of this sorting procedure have been published elsewhere (21).

More than 95% of the IgD^+ cells obtained in this manner were fluorescent on visual examination by using fluorescence microscopy. Direct staining of these cells with a rhodaminated-specific anti-IgM antiserum and subsequent FACS analysis showed that greater than 95% of IgD^+ cells also carry surface IgM. The isolated IgD^- population was stained with an antiserum specific for T cells and found to contain mainly T cells.

The Expression of IgD on the Primary Responsive Lymphocyte. IgD^+ and IgD^- lymphocytes were prepared as outlined above. Mixtures of stained but unseparated or separated fractions with added T cells were transferred into X-irradiated BALB/cN mice along with BRBC, and the splenic PFC response that arose 7 days later was assessed. About half of the unseparated normal spleen cells are IgD^+, therefore equal numbers of IgD^- and IgD^+ cells were transferred.

The results from the above experiment are shown in Table III. IgD^+ lymphocytes supplemented with T cells gave rise to a response approximately equivalent to unseparated spleen cells supplemented with T cells. IgD^- cells supplemented with T cells did not generate a PFC response. This experiment is representative of four similar studies that were performed. (Similar results were also obtained on transfer of IgD^+ and IgD^- SJL cells into SJL recipients.) Thus IgD^+ cells give rise to a primary response to an erythrocyte antigen. Since the IgD^- lymphocyte fraction contained only about 5-10% B cells we cannot say that IgD^- B cells are totally unable to participate in T-dependent primary immune responses; rather, we conclude that most, if not all, BRBC primary-reactive B cells reside in the IgD^+ lymphocyte pool.

The Expression of IgD on IgG Memory Cells. The results of adoptive transfers with primed B cells are presented in Table IV.

Both the isolated IgD^+ cells and the isolated IgD^- cells from DNP-primed mice, when supplemented with KLH-primed spleen cells, generated substantial IgG PFC responses. Thus the IgG memory pool present 6 wk after one exposure to antigen is heterogeneous, with memory being found in both IgD^+ and IgD^- fractions. These data are representative of results obtained in three similar

TABLE IV

Expression of IgD on IgG Memory Cells

DNP-Primed B Cells[*] (5×10^6)	DNP-KLH[‡]	Anti-DNP PFC ($\times 10^3$)/spleen
		Developed
Stained unseparated spleen	+	160
Stained unseparated spleen	–	4.7
IgD$^+$ fraction	+	50
IgD$^-$ fraction	+	44
--	+	<0.1

All recipient mice received 10^7 KLH-primed SJL spleen cells harvested from animals primed 6 wk previously with 100 μg alum-precipitated KLH and *B. pertussis* organisms. SJL spleen cells were stained with SJA anti-Ig-5b serum and F*b anti-a allotype serum, then sorted into bright IgD$^+$ (top 37%) and dull IgD$^-$ (bottom 35%) fractions. Recipients were X-irradiated (650 rads) BALB/c mice. The PFC response was assessed 7 days after transfer.

[*] SJL mice were primed 6 wk previously with 100 μg alum-precipitated DNP-KLH together with 2×10^9 killed *B. pertussis* organisms.

[‡] Where indicated (+) mice were challenged with 10 μg aqueous DNP-KLH.

experiments using the allogeneic transfer system and one experiment in which a syngeneic transfer was performed.

In the above experiments, the proportion of IgD$^+$ memory cells comprised approximately half the memory cell population; however, in other experiments with donors taken later than 6 wk after priming, the proportion of IgD$^+$ memory cells in the memory population was consistently lower, apparently decreasing with time after antigenic exposure (S. Black, unpublished observations).

The Avidity of PFC Generated from IgD$^+$ and IgD$^-$ Memory Cells. As the IgG memory pool arising after one exposure to antigen proved to be heterogeneous, we investigated the avidity of the DNP-PFC populations arising from IgD$^+$ and IgD$^-$ memory cells. The experimental protocol used was identical to that described in the preceding section. The avidity of DNP-PFC was measured in the Cunningham assay by inhibition with graded concentrations of ϵ-DNP-lysine included in the plaquing chamber. The results of this experiment are presented in Table V and are representative of two such experiments performed.

Data in Table V confirms that IgG memory is carried by both IgD$^+$ and IgD$^-$ B lymphocytes in mice primed 6 wk previously with DNP-KLH. There are differences, however, in the avidity of the PFC populations arising from each of these B-cell groups. The data in this table are presented as the percentage of the DNP-PFC response that was inhibited between the indicated concentration ranges of ϵ-DNP-lysine. Thus, the greater the percentage of inhibition obtained with low concentrations of ϵ-DNP-lysine present, the more PFC of high avidity were present in the population analyzed.

Stained but unseparated DNP-primed SJL spleen cells when supplemented with KLH-primed spleen cells gave rise to a PFC response of intermediate avidity. Most PFC were inhibited with between 10^{-5} and 10^{-8} M ϵ-DNP-lysine present in the plaquing chamber. These account for 67% of the total PFC

TABLE V

Avidity of DNP-PFC Generated by IgD$^+$ or IgD$^-$ Memory Cells

ε-DNP-lysine (-log$_{10}$ M) in PFC Chamber	% Inhibition of DNP-PFC response within each concentration range		
	IgD$^+$	IgD$^-$	Whole spleen
<5	8	3	7
5–6	28	9	22
6–7	39	24	24
7–8	12	22	21
8–9	6	22	11
9–10	4	12	9
>10	3	8	6

5 × 10^6 DNP-primed cells (IgD$^+$, IgD$^-$, and whole spleen stained but unseparated) and 10^7 KLH-primed spleen cells were transferred with 10 μg DNP-KLH into X-irradiated (650 rads) BALB/c recipients. Spleens were harvested and plaqued against DNP-BRBC on day 7 after transfer. ε-DNP-lysine was incorporated into the suspending medium at the indicated concentration before placing cells into the PFC chamber. Results of the inhibition assay are expressed as percentage of the normal PFC response which is inhibited between the indicated concentration ranges of ε-DNP-lysine. The developed PFC responses (without inhibitor present) obtained from each group were as follows, expressed as (×10^3)/spleen: IgD$^+$, 120; IgD$^-$, 103; unseparated, 215. Further statistical details are given in Materials and Methods.

response. The remaining PFC were inhibited over the entire range of inhibitor concentrations. IgD$^+$ B cells supplemented with carrier-primed T cells gave rise to PFC that were mainly inhibitable by concentrations of ε-DNP-lysine between 10^{-5} and 10^{-7} M. Thus the avidity of this response was a little lower than that of the unseparated population of spleen cells. In contrast IgD$^-$ B cells in combination with carrier-primed spleen cells generated a PFC response of higher avidity than the unseparated spleen cell preparation. The majority (88%) of these PFC were inhibited with between 10^{-6} and 10^{-9} M ε-DNP-lysine. Since increase in avidity is associated with maturation of the immune response (20), the substantially higher avidity of the IgD$^-$ component of the B-cell memory population suggests that these cells represent a later stage of memory development.

The Expression of IgD on IgG Memory Cells after Several Exposures to Antigen. The data presented in the preceding section suggested that IgD$^-$ IgG memory cells were more mature than IgD$^+$ IgG memory cells. To confirm this possibility, mice were exposed to more than one dose of antigen before their spleen cells were harvested, FACS-separated into IgD$^+$ and IgD$^-$ fractions, and these populations analyzed for their capacity to transfer an adoptive IgG anti-DNP-PFC response. Mice were primed with DNP-KLH 6 wk before harvesting of spleens. These animals were then split into two groups. The first set of mice was analyzed immediately for the amount of IgG memory carried in IgD$^-$ and IgD$^+$ fractions. The second group was re-exposed to 10 μg aqueous DNP-KLH and rested for an additional 4 wk. This group was then divided. Some mice were analyzed for the amount of IgG memory carried by IgD$^+$ and IgD$^-$ B lymphocyte populations and the rest were again exposed to 10 μg aqueous

TABLE VI

Expression of IgD on IgG Memory Cells after Exposure(s) to Antigen

DNP-Primed B Cells*	No. of antigen‡ injections	Developed Anti-DNP PFC ($\times 10^3$)/spleen
Stained§ unseparated spleen	1	250
IgD$^+$ fraction	"	160
IgD$^-$ fraction	"	101
Stained‖ unseparated spleen	2	190
IgD$^+$ fraction	"	21
IgD$^-$ fraction	"	140
Stained¶ unseparated spleen	3	350
IgD$^+$ fraction	"	<0.1
IgD$^-$ fraction	"	320

* 5 × 10^6 B cells along with 10^7 KLH° spleen cells and 10 μg aqueous DNP-KLH were transferred i.v. into each recipient mouse. In the absence of antigen no response was obtained.

‡ 1, primed with DNP-KLH 6 wk previously. 2, primed with DNP-KLH 10 wk previously and boosted with DNP-KLH at 6 wk later. 3, primed with DNP-KLH 14 wk previously, boosted with DNP-KLH at 6 and 10 wk after priming.

§ 43% cells stained; 28% selected as IgD$^+$; 31% as IgD$^-$.

‖ 37% cells stained; 22% selected as IgD$^+$; 29% as IgD$^-$.

¶ 39% cells stained; 26% selected as IgD$^+$; 31% as IgD$^-$.

DNP-KLH. After another 4 wk, IgD$^+$ and IgD$^-$ cells harvested from these animals were probed for their capacity to transfer an IgG PFC response.

The results of these experiments are presented in Table VI. This experiment was performed twice with similar results. Each time the DNP-primed donor mice were exposed to antigen a change occurred in the degree of IgG memory carried by IgD$^+$ and IgD$^-$ populations of B lymphocytes. After three exposures to antigen, IgG memory became limited to the IgD$^-$ population of B lymphocytes.

These results suggest that IgD$^+$ memory cells arise early in the immune response and become converted to IgD$^-$ memory cells with repeated antigenic exposure; however, establishment of this sequence requires direct examination of isolated IgD$^+$ cells to determine their ability to give rise to IgD$^-$ memory cells. Preliminary data from such double transfer experiments is in agreement with an IgD$^+$ to IgD$^-$ switch, but definitive data on this point has not yet been obtained (S. Black, unpublished observations).

The Expression of IgD on Primary IgM and IgG PFC. SJL mice were injected with 5 × 10^7 washed BRBC and spleen cells were harvested 5 days later. These cells were separated into IgD$^+$ and IgD$^-$ fractions on the FACS-II. Of each population of lymphocytes 2 × 10^5 cells were plaqued against BRBC with or without facilitating antiserum and the distribution of direct and developed PFC noted. The results from this experiment are presented in Table VII and are representative of four such experiments conducted. Table VII

TABLE VII

The Expression of IgD on Primary IgM and IgG PFC

Cells	Anti-BRBC PFC*	
	Direct PFC/(2×10^5)	Developed PFC/(2×10^5)
IgD$^+$	80	3
IgD$^-$	3	204

* A day 5 anti-BRBC-PFC response after injection of 5×10^7 washed BRBC.

TABLE VIII

The Expression of IgD on Secondary IgG PFC

Cells	Anti-DNP PFC*
	Developed PFC/(2×10^5)
IgD$^+$	20
IgD$^-$	320

* A day 7 anti-DNP-PFC response generated by DNP-KLH primed SJL mice after challenge with 10 μg aqueous DNP-KLH.

shows that primary IgM anti-BRBC PFC have IgD on their membrane whereas IgG PFC do not.

The Expression of IgD on Secondary IgG PFC. SJL mice primed with DNP-KLH were boosted with aqueous DNP-KLH and 7 days later their spleens were harvested. These cells were separated into IgD$^+$ and IgD$^-$ fractions. Both fractions were then assayed with DNP-BRBC in the Cunningham plaque assay. Facilitating antiserum was included as no direct PFC were generated. The data from this experiment are presented in Table VIII. Most IgG PFC were IgD$^-$. The small number of IgG PFC (4-5%) that fell in the IgD$^+$ fraction probably represents contamination of the IgD$^+$ cells with a few IgD$^-$ cells. These data are similar to results obtained in three separate experiments.

Discussion

We have shown that IgD is present on the surface of unprimed B cells and early B memory cells but not on mature memory B cells. IgD is also present on the surface of IgM PFC but not on IgG PFC even when the IgG PFC arise as a result of a primary immune response.

The proportion of IgD$^+$ IgG memory cells is highest in recently primed animals (approximately 6 wk). If these primed animals are boosted, the proportion of IgG memory B cells found in the IgD$^+$ population of spleen cells decreases and most of these memory cells are found among IgD$^-$ B cells. These data suggest that IgD marks the early cells in the B-memory cell developmental pathway.

The lower avidity of the antibody produced by progeny of IgD$^+$ memory cells as compared with the avidity of the antibody produced by progeny of IgD$^-$ memory cells strongly supports the assertion that IgD$^-$ memory cells are the most mature. This hypothesis is further supported by the observation that all

IgG PFC in a secondary response lack surface IgD, hence when a cell enters the final stages of development to an IgG PFC it ceases to have surface IgD.

The above data confirm and extend the work of Coffman and Cohn (7) and Zan-bar et al. (8, 9) on the expression of IgD on B memory cells committed to generating an IgG secondary response. They also support the findings of Coffman and Cohn (7) that IgM-IgD lymphocytes participate in a primary immune response to a T-dependent antigen, and are consistent with previous observations on B-cell development as well (4, 5).

When the evidence gathered in these various studies is taken in concert with the evidence presented in this publication, the outlines of the B-cell maturation pathway, in which the expression of different Ig isotypes marks successive stages of maturation, can be drawn as follows: the first B cells to arise have IgM on their surface. These cells can respond to T-independent antigenic stimulus (7) but tolerance is easily induced if they are exposed to a T-dependent antigen (22). A second population of B cells later appears. These cells express surface IgM and IgD and are possibly progeny of the IgM only cells. On exposure to a T-independent antigen, at least a component of this IgM-IgD population will differentiate to IgM PFC (7–9); if a T-dependent antigen is used a component of this population will differentiate to IgM and IgG PFC. At present it is not known if the same cells in this population respond to both T-dependent and T-independent antigens.

Exposure to antigen induces formation of a memory B-cell population. The memory cell population is heterogeneous with respect to expression of surface IgD. The first memory cells appear to be largely IgD$^+$. With time and exposure to antigen, the memory population becomes IgD$^-$. It is likely, although definitive evidence is still lacking, that the IgD$^+$ memory cell gives rise to the IgD$^-$ memory cell.

Definitive data on the other isotypes expressed on the IgD$^+$ and IgD$^-$ memory B cells is still lacking. Most splenic memory cells carry surface IgG that indicates their class and allotype commitment (10, 11). This includes at least a significant proportion of the IgD$^+$ memory cells (7). Again, a proportion of memory cells carry surface IgM (9). It is reasonable to assume that early memory cells first express IgM and IgD, then express IgG in addition, and finally lose the IgM and IgD isotypes from their surface as the response matures (i.e., with time and exposure to antigen).

Summary

B lymphocytes capable of generating primary IgM and IgG plaque-forming cells (PFC) responses to burro erythrocytes have surface IgD, as do primary IgM PFC. IgG memory cells arising after one injection of antigen are divided into two groups, one of which expresses surface IgD while the other has no detectable membrane IgD. PFC generated from the IgG memory cells lacking surface IgD show a higher average avidity than those arising from IgD-positive IgG memory cells, indicating that mature IgG memory cells do not have surface IgD. After more than one injection of antigen, few, if any, IgG memory cells have surface IgD. IgG PFC arising in primary or secondary immune response lack membrane-bound IgD. These data provide the outlines for a B-cell

maturation pathway in which IgD marks unprimed and early memory B cells and is lost in mature memory cells.

Studies presented here were conducted by isolating IgD$^+$ and IgD$^-$ cells with the fluorescence-activated cell sorter and functional testing of the isolated populations in adoptive transfer experiments.

We thank Ms. Sandra Scaling for providing excellent technical assistance throughout this project, Ms. Jean Anderson for assuming considerable responsibility in the preparation of this manuscript, and Ms. Lee Herzenberg for providing both critical evaluation and editorial assistance as this work was being prepared for publication. We also wish to express our appreciation to Mr. Eugene Filson for his invaluable aid with the FACS separations.

Received for publication 25 October 1977.

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IMMUNOSUPPRESSIVE FACTORS FROM LYMPHOID CELLS OF NONRESPONDER MICE PRIMED WITH L-GLUTAMIC ACID^{60}-L-ALANINE^{30}-L-TYROSINE^{10}

IV. Lack of Strain Restrictions Among Allogeneic, Nonresponder Donors and Recipients*

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Antibody responses to the synthetic terpolymer L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT)[1] in mice are controlled by *H-2*-linked-immune response (Ir) genes (1). Injection of nonresponder mice, $H\text{-}2^{p,q,s}$, with GAT fails to stimulate GAT-specific plaque-forming cell (PFC) responses but does activate suppressor T cells (Ts cells) specific for GAT (GAT-Ts cells) (1, 2). GAT-Ts cells inhibit the development of GAT-specific IgG PFC responses by syngeneic mice immunized with GAT complexed to the carrier methylated bovine serum albumin (GAT-MBSA) in vivo and in vitro.

Extracts prepared from lymphoid cells of GAT-primed nonresponder, but not responder, mice specifically inhibit the development of antibody responses to GAT-MBSA by normal syngeneic nonresponder mice in vivo and in vitro (3). The suppressive factor in these extracts is a protein of approximately 45,000 mol wt (4) which can be extracted from purified T cells but not B cells, and is therefore referred to as GAT-TsF (5). GAT-TsF specifically binds to insolubilized GAT and can be eluted with 3.0 M KCl. Despite this affinity GAT-TsF does not bear μ, γ_1, γ_{2a}, γ_{2b}, or α-heavy chain or κ-light chain constant region determinants (4). However, GAT-TsF is absorbed by insolubilized antisera specific for determinants encoded by the *I* region of the *H-2* gene complex (4), and therefore belongs to the same class of immunosuppressive molecules described by Tada et al. (6). In the crude lymphoid cell extracts, GAT-TsF is associated with an immunologically detectable fragment of GAT (4). However, the amount of GAT associated with GAT-TsF is, by itself, insufficient to cause suppression (4). Furthermore, GAT-TsF purified by absorption to and elution from insolubilized

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* Supported by U. S. Public Health Service grant AI-13987 from the National Institute of Allergy and Infectious Diseases and Biomedical Research Support grant RR05491 to The Jewish Hospital of St. Louis from the Division of Research Resources, National Institutes of Health.

[1] *Abbreviations used in this paper:* GAT, random terpolymer of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}; GAT-MBSA, GAT complexed to methylated bovine serum albumin; GAT-SRBC, GAT complexed to sheep erythrocytes; GAT-Ts cells, GAT-specific suppressor T cells; GAT-TsF, GAT-specific suppressor T-cell factor; H-2, murine major histocompatibility complex; Ir gene, immune response gene(s); $M\phi$, macrophage(s); PFC, plaque-forming cell(s); Ts cells, suppressor T cell(s).

GAT no longer contains detectable GAT; presumably, the fragment of GAT complexed to GAT-TsF is displaced by insolubilized GAT. Nevertheless, purified GAT-TsF bears Ia antigen and is suppressive, suggesting that the original GAT fragment associated with GAT-TsF is not essential for suppression. However, this observation does not establish that antigen is unnecessary for specific suppressive activity since the assay for activity requires the presence of GAT-MBSA.

GAT-TsF has been detected in extracts from two nonresponder strains of mice (DBA/1 and A.SW) (3). In addition, an extract from GAT-primed DBA/1 mice inhibits the response to GAT-MBSA by spleen cells from a histoincompatible nonresponder strain of mice (A.SW) but not a histoincompatible responder strain (C57BL/6) (5).

The observations that GAT-TsF is associated with a fragment of GAT in crude extracts and that GAT-TsF from one strain of nonresponder mice could suppress another histoincompatible nonresponder strain of mice distinguish this molecule from the carrier-specific TsF described by Tada et al. (6, 7). Carrier-specific TsF is not associated with antigen and suppresses immune responses only when donors and recipients are syngeneic at I-J.

To determine whether the suppression by GAT-TsF across histocompatibility barriers was peculiar to the two strains of mice tested or was a general characteristic of the GAT system, the following experiments were performed. GAT-TsF from mice bearing the three nonresponder $H\text{-}2^{p,q,s}$ haplotypes on a variety of non-H-2 backgrounds was prepared and analyzed for specific suppressor activity on in vitro PFC responses to GAT-MBSA by spleen cells from mice that were syngeneic or allogeneic with the GAT-TsF donor.

Materials and Methods

Mice. C57BL/10 (H-2^b), P/J (H-2^p), DBA/1 (H-2^q), SWR (H-2^q), and SJL (H-2^s) mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. A.SW (H-2^s) and B10.S (H-2^s) mice were bred in the animal facilities at The Jewish Hospital by brother-sister mating of the offspring of breeder pairs obtained from The Jackson Laboratory. B10.P (H-2^p) mice were kindly provided by Dr. Donald Shreffler, Washington University School of Medicine. Mice used were 2- to 8-mo-old and were maintained on laboratory chow and water ad lib.

Antigens. GAT, mol wt 45,000, was purchased from Miles Laboratories, Inc., Miles Research Products, Elkhart, Ind. MBSA (Worthington Biochemical Corp., Freehold, N. J.), sheep erythrocytes (SRBC), GAT and GAT-MBSA were prepared as described previously (1).

Preparation of Cell-Free Extracts. Cell-free extracts of lymphoid cells from GAT-primed mice were prepared as previously described (3). Briefly, mice were injected i.p. with 10 μg GAT in Maalox (Wm. H. Roher, Inc., Fort Washington, Pa.). These mice were sacrificed 3-7 days later, and single cell suspensions of spleens and thymuses were prepared, pooled, washed twice in Hanks' balanced salt solution, and resuspended to 6 × 10^8 cells/ml in Eagle's minimum essential medium supplemented with 4 mM Hepes, 2 mM L-glutamine and 50 U each penicillin and streptomycin (Microbiological Associates, Walkersville, Md.). The cells were sonicated at 7°C in a Soncifier Cell Disruptor model W-140-E (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.). The lysate was centrifuged at 40,000 g for 30 min and the supernate collected and stored at −80°C.

Antisera. Alloantisera specific for the (I-B, -J, -E, -C; S; G)^b (anti-I^b) and (I, S, G)^s (anti-I^s) regions of the *H-2* complex were donated by Dr. Shreffler. Anti-I^b was prepared by immunizing [B10.A (2R) × C3H.Q]F_1 with B10.A (4R) lymphoid cells. Anti-I^s was prepared by immunizing (A.TL × A.TFR3)F_1 with A.TH lymphoid cells. Anti-GAT sera was obtained from rabbits 7 days after the second subcutaneous immunization with 1 mg GAT in complete Freund's adjuvant.

Preparation of Immunoadsorbents. GAT-Sepharose 4B: GAT was coupled to amino hexyl-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N. J.) as previously described (4). Briefly, GAT was dissolved in water and coupled to the beads with 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide (Story Chemical Corp., Muskegon, Mich.). The coupling conditions were empirically adjusted such that 1-2 mg GAT was coupled to 1 ml of packed Sepharose.

Anti-GAT, anti-I^b, and anti-I^s: all sera were heat inactivated at 56°C for 30 min and the globulin fractions were prepared by precipitation with 50% ammonium sulfate. The precipitates were dissolved in and extensively dialyzed against 0.5 M NaCl containing 0.1 M $NaHCO_3$. The globulin fractions of these sera were coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals) according to the manufacturers directions. Coupling conditions were adjusted to obtain 2 mg of protein per ml packed Sepharose.

Use of Immunoadsorbents. Extracts were diluted 1:10, applied to immunoadsorbent columns, and reacted at 4°C for 1-2 h. The unbound material was collected, the columns extensively washed with Hanks' balanced salt solution, and bound material was eluted with 3.0 M KCl. KCl was removed from the eluates by filtration through Sephadex G-25 (Pharmacia Fine Chemicals) (4).

Cell Cultures, Assay of GAT-TsF and PFC Assay. Replicate 1-ml cultures containing 8-10 × 10^6 spleen cells were established according to the modifications of the Mishell-Dutton system used in our laboratory (8) and dilutions of GAT-TsF and 5 μg GAT as GAT-MBSA or SRBC was added at culture initiation. PFC responses were assayed 5 days later using SRBC or GAT coupled to SRBC (GAT-SRBC) as indicator cells. The activity of GAT-TsF is expressed at S_{50} U/ml which is defined as the inverse of the final dilution of extracts that causes 50% suppression of the PFC response. If no suppressive activity is observed at the lowest dilution tested, the titer is expressed as less than the inverse of the dilution. Conversely, if the 50% end point is not obtained at the highest dilution tested, the titer is recorded as greater than that dilution.

Results

GAT Stimulates Production of GAT-TsF in Nonresponder Strains of Mice Bearing $H\text{-}2^{p,q,s}$ Haplotypes. Extracts prepared from lymphoid cells of GAT-primed B10.P (H-2^p), DBA/1 (H-2^q) and A.SW (H-2^s) mice were diluted and added to cultures containing spleen cells syngeneic with the GAT-TsF donors stimulated with SRBC or GAT-MBSA (Fig. 1). Control cultures containing B10.P, DBA/1, or A.SW spleen cells stimulated with GAT-MBSA in the absence of any extracts developed 340, 1,375, and 800 GAT-specific IgG PFC/culture, respectively. Although not shown, none of these extracts suppressed the IgM or IgG PFC responses to SRBC at dilutions of 1/400 or greater. Clearly, GAT-TsF can be produced by GAT-primed mice bearing any one of the three nonresponder haplotypes. Repeated titrations of a given extract have been remarkably consistent although some variation in suppressive activity from one batch to another has been observed (3).

Suppression of Immune Responses of Nonresponder Mice by Allogeneic GAT-TsF. Suppression of GAT-MBSA responses by spleen cells from five nonresponder strains of mice by an H-2^q GAT-TsF is shown in Table I. PFC responses to GAT-MBSA by nonresponder mice bearing H-2^p (P/J) and H-2^s (SJL) haplotypes but not responder mice bearing H-2^b (C57BL/10) haplotype were specifically suppressed by H-2^q (DBA/1 or SWR) GAT-TsF. Furthermore, responses by spleen cells from congenic strains (A.SW, B10.S and B10.P) bearing nonresponder *H-2* haplotypes were also suppressed by DBA/1 GAT-TsF regardless of the origin of non-*H-2* genes.

Extracts from GAT-primed B10.P and A.SW mice specifically suppressed PFC responses by B10.P, DBA/1, and A.SW spleen cells (Table II). The data in Tables I and II demonstrate that GAT-TsF from donors bearing any one of the three nonresponder haplotypes reciprocally suppressed recipients bearing any one of the three nonresponder haplotypes. In this experiment the activity of

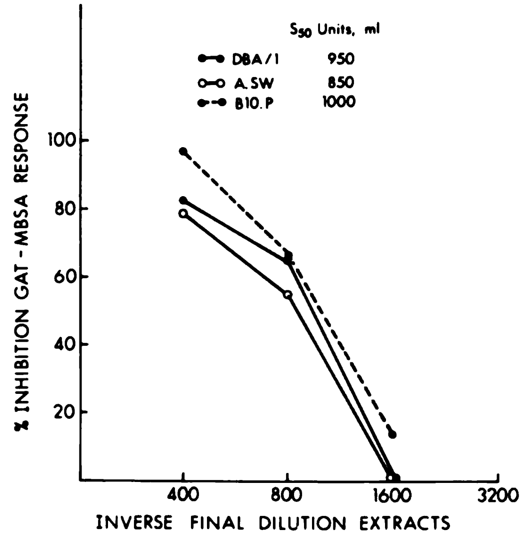

FIG. 1. In vitro analysis of lymphoid cell extracts from GAT-primed DBA/1, A.SW, and B10.P mice. All extracts were prepared from pooled thymus and spleen cells (6×10^8 cells/ml) from mice primed i.p. 3 days earlier with 10 μg GAT in Maalox. Extracts were diluted and added to spleen cells from normal syngeneic mice at culture initiation. IgG PFC responses stimulated by GAT-MBSA and SRBC (not shown) were assayed after 5 days. Suppression is expressed as S_{50} U/ml which is the inverse of the dilution of extract that causes 50% inhibition of the PFC response by control cultures.

GAT-TsF from A.SW mice was somewhat lower than that from B10.P or DBA/1 mice (Table I). Multiple extracts prepared from A.SW mice demonstrate fluctuations in levels of activity and frequently contain less activity than H-2^p or H-2^q extracts (data not shown).

The data in Table I demonstrated that non-*H-2* genes did not detectably alter the susceptibility of recipient nonresponder spleen cells to a given GAT-TsF. Furthermore, nonresponder mice bearing the *H-2^p* or *H-2^s* haplotypes on a background of responder (C57BL/10 or A) genes were capable of producing GAT-TsF (Fig. 1 and Table II), suggesting that non-*H-2* genes did not control production of GAT-TsF. The possibility that non-*H-2* genes might subtly regulate production of GAT-TsF was examined in a greater detail by comparing suppressive activities of extracts from GAT-primed mice bearing the *H-2^s* haplotype and various non-*H-2* genes on the immune response by DBA/1 spleen cells. In the experiment shown in Table III, no detectable differences were found in the level or specificity of suppression mediated by extracts of lymphocytes prepared simultaneously from age matched SJL, A.SW, or B10.S mice 3 days after priming with GAT, suggesting that responder non-*H-2* genes do not regulate production of GAT-TsF.

Is Suppression of Responses by Syngeneic and Allogeneic Spleen Cells Mediated by the Same Molecule? Although GAT-TsF mediated suppression of PFC responses by syngeneic and allogeneic recipient spleen cells which was antigen-specific, it is still possible that suppression of responses by allogeneic spleen cells is mediated by a moiety distinct from that active in syngeneic recipient spleen cells. We have previously demonstrated that suppression of

TABLE I

Suppression of Responses by Nonresponder Mice by GAT-TsF from H-2^{q} Mice

Spleen cells*	*H-2*	SRBC	GAT-MBSA
		S_{50} *U/ml*‡	
DBA/1	*q*	<400	1,600§
P/J‖	*p*	<400	1,600
SJL	*s*	<400	1,200
C57BL/10¶	*b*	<400	400
A.SW	*s*	<400	1,500
B10.S	*s*	<400	1,500
B10.P	*p*	<400	1,500

* Spleen cells cultured at 8–10 × 10^{6} cells/ml.

‡ Inverse of the final dilution GAT-TsF causing 50% suppression of the PFC response.

§ Data in this Table are compiled from several experiments in which an H-2^{q} GAT-TsF was titrated on the immune response by spleen cells from DBA/1 and other nonresponder strains. The activity of GAT-TsF in individual experiments was normalized to the average activity on DBA/1 responses of 1,600 S_{50} U/ml.

‖ Spleen cells from P/J mice were treated with GAT-TsF from SWR (H-2^{q}) mice.

¶ C57BL/10 mice are responders to GAT.

TABLE II

Suppression of Responses by Nonresponder Mice of Different Haplotypes by GAT-TsF from H-2^{p} or H-2^{s} Mice in Vitro

Spleen cells‡		H-2^{p} GAT-TsF		H-2^{s} GAT-TsF	
Strain	*H-2*	SRBC	GAT-MBSA	SRBC	GAT-MBSA
			s_{50} *U/ml**		
B10.P	*p*	<400	1,000	<400	1,000
DBA/1	*q*	<400	2,100	<200	930
A.SW	*s*	<400	1,600	<400	850

* Inverse final dilution of extracts from B10.P (H-2^{p}) or A.SW (H-2^{s}) mice causing 50% suppression of the immune response.

‡ Spleen cells cultured at 8–10 × 10^{6} cells/ml.

responses in DBA/1 spleen cells by DBA/1 GAT-TsF is mediated by a factor that bears determinants encoded by the *I* region of the H-2^{q} complex (4). Therefore, a crude extract from GAT-primed A.SW mice and the extract after absorption with insolubilized anti-I^{b} or anti-I^{s} alloantisera were tested for suppression of responses by spleen cells from DBA/1 and A.SW mice (Table IV). Titration of these materials on syngeneic spleen cell responses indicated that H-2^{s} GAT-TsF was absorbed by an alloantisera reactive against I^{s} but not I^{b}. Similarly, GAT-specific suppression of the PFC response by DBA/1 spleen cells occurred when these cells were incubated with the untreated H-2^{s} GAT-TsF and the extract which was passed over an anti-I^{b} immunoadsorbent. Unbound material from an anti-I^{s} immunoadsorbent failed to suppress the DBA/1 PFC response.

Suppression of PFC Responses in Syngeneic and Allogeneic Recipient Spleen Cells by Crude and Purified GAT-TsF. In addition to suppressing responses

TABLE III

Immune Responses by DBA/1 Spleen Cells are Suppressed by Extracts from Mice Bearing the H-2^s Haplotype and Various Non-H-2 Genes

Source GAT-TsF	*H-2* Haplotype of donor	SRBC	GAT-MBSA
		S_{50} *U/ml**	
DBA/1	*q*	<400	1,450
SJL	*s*	<400	1,150
A.SW	*s*	<400	1,350
B10.S	*s*	<400	1,500

* See Table I.

TABLE IV

Suppressive Moiety in GAT-TsF from A.SW Mice Bears Antigens Encoded by the H-2 Complex

A.SW GAT-TsF Treatment	A.SW		DBA/1	
	SRBC	GAT-MBSA	SRBC	GAT-MBSA
		S_{50} *U/ml**		
None	<400	1,150	<200	930
Absorption with anti-I^b	480	2,100	<200	1,600
Absorption with anti-I^s	410	<400	<200	<200

* See Table I.

TABLE V

Suppression of Responses by Syngeneic and Allogeneic Spleen Cells with Crude and Purified GAT-TsF

Group	Treatment of DBA/1 GAT-TsF	DBA/1	B10.S
		S_{50} *U/ml**	
A	None	3,500	3,800
B	Absorbed by Anti-GAT-Sepharose	500	NT‡
C	Absorbed by GAT-Sepharose	<400	NT
D	Eluted from GAT-Sepharose (GAT-TsFP)	5,600	5,800
E	GAT-TsFP Absorbed by Anti-GAT-Sepharose	5,800	NT

* Suppressive activity was titrated on PFC responses to GAT-MBSA. Although not shown the immune responses to SRBC by DBA/1 or B10.S spleen cells were not suppressed by any of the GAT-TsF fractions tested at dilutions of $\geq 1/400$.

‡ Not tested.

to GAT-MBSA by allogeneic nonresponder mice, GAT-TsF differs from the carrier specific TsF described by Tada et al. (7) in that GAT-TsF is associated with a fragment of immunologically recognizable antigen. An experiment was designed to determine whether association with a GAT fragment accounted for suppression of allogeneic recipient mice by GAT-TsF (Table V). GAT-TsF from DBA/1 mice was specifically suppressive for both DBA/1 and B10.S GAT-MBSA responses (group A). Association of immunoreactive GAT with GAT-TsF in crude extracts was verified by absorbing the crude extract with insolubilized rabbit anti-GAT globulin (group B) which reduced the S_{50} titer by 85%. GAT-TsF was also bound by GAT-Sepharose (group C) and was eluted from GAT-Sepharose with 3.0 M KCl (group D). The apparent increase in activity of eluted GAT-TsF is frequently observed and is most likely due to removal of nonspecific enhancing material present in crude extracts (3). Purified GAT-TsF, eluted from GAT-Sepharose, is no longer absorbed by rabbit anti-GAT-Sepharose (group E). Nevertheless, the PFC response by B10.S spleen cells to GAT-MBSA was suppressed by crude as well as purified DBA/1 GAT-TsF (group D). This observation suggests that the fragment of GAT associated with GAT-TsF in crude extracts is not responsible for the ability of GAT-TsF to suppress anti-GAT-MBSA PFC responses across histocompatibility barriers.

Discussion

These experiments demonstrate that lymphoid cell extracts from GAT-primed $H\text{-}2^p$ mice, like extracts from mice bearing the other nonresponder haplotypes, $H\text{-}2^q$ and $H\text{-}2^s$ (3), contain GAT-specific immunosuppressive factors (Fig. 1). In addition, GAT-specific suppression of responses by allogeneic ($H\text{-}2^s$) nonresponder spleen cells by DBA/1 ($H\text{-}2^q$) GAT-TsF has been confirmed (5) and extended to other allogeneic nonresponder recipient spleen cells (Table I). Suppression by allogeneic GAT-TsF is not restricted by the presence of responder non-*H-2* genes in recipients, since DBA/1 GAT-TsF suppressed responses by spleen cells from SJL ($H\text{-}2^s$) and the congeneic strains of A.SW ($H\text{-}2^s$) and B10.S ($H\text{-}2^s$) equally (Table I). More importantly, all nonresponder strains of mice, regardless of the source of non-*H-2* genes are suppressed by GAT-TsF from all other strains of mice bearing the nonresponder $H\text{-}2^{p,q,s}$ haplotypes (Tables I and II). Extracts prepared from mice bearing the $H\text{-}2^s$ haplotypes on non-*H-2* gene backgrounds of SJL, A, or C57BL/10 suppressed the GAT-MBSA response of DBA/1 spleen cells with no detectable differences in antigen specificity or level of activity (Table III) suggesting that non-*H-2* genes do not modulate the production of GAT-TsF.

Previously published data, demonstrated that DBA/1 GAT-TsF bears determinants encoded by the *I* region of the *H-2* gene complex (7). Absorption of A.SW GAT-TsF by insolubilized alloantisera specific for *I* subregion of the $H\text{-}2^s$, but not $H\text{-}2^b$, gene complex demonstrates that GAT-TsF from a second nonresponder strain of mice bears determinants that map between the *H-2K* and *H-2D* regions (Table IV). Furthermore, the substance in A.SW GAT-TsF that suppressed responses by allogeneic recipient spleen cells also contains determinants encoded by the $H\text{-}2^s$ gene complex (Table V). This observation suggests, but does not prove, that suppression of responses by syngeneic and allogeneic spleen cells is mediated by the same molecules. The conclusion that suppression by GAT-TsF does not require that donors and recipients are identical at the *I-J* subregion is tenuous since the *I-J* subregions of $H\text{-}2^p$ and $H\text{-}2^q$ have not yet been identified by recombination. Thus, the argument could be made that $I\text{-}J^p$, $I\text{-}J^q$, and $I\text{-}J^s$ genes encode molecules that contained shared specificities and that suppression by GAT-TsF across histocompatibility barriers actually represents cross-reactivity. Indirect evidence against this possibility has been obtained by Thèze et al. who have shown that suppressive factors obtained from BALB/c and B10.BR mice primed with the copolymer L-glutamic-acid^{50}-L-tyrosine^{50} (GT) bear distinct alloantigens encoded by the *I-J* subregion and nevertheless reciprocally suppress GT-MBSA responses (9).

One mechanism by which GAT-TsF suppresses GAT-MBSA PFC responses is by stimulation of GAT-Ts cells in the recipient (10). In addition, crude extracts containing GAT complexed to GAT-TsF stimulates GAT-Ts cells in recipient mice whereas purified GAT-TsF containing no detectable GAT does not (11). Furthermore, the addition of small amounts of GAT, in concentrations too low to stimulate Ts cells independently, to purified GAT-TsF restores the capacity to stimulate GAT-Ts cells (11). This observation raised the possibility that GAT-TsF complexed to a fragment of GAT (as it is found in crude extracts) might suppress across histocompatibility barriers by virtue of stimulating autologous GAT-Ts cells in the recipient and thereby masking potential restrictions between donors and recipients. To test this possibility, crude DBA/1 GAT-TsF was analyzed for the presence of immunoreactive GAT, and another portion of this extract was absorbed to GAT-Sepharose and purified GAT-TsF that was subsequently eluted from GAT-Sepharose was re-examined for the presence of a fragment of GAT. Each of these fractions were assayed for functional activity (Table V). Crude GAT-TsF containing GAT and purified GAT-TsF containing no detectable GAT determinants suppressed allogeneic as well as syngeneic responses (Table V). Thus, the ability of GAT-TsF to suppress across histocompatibility barriers cannot be attributed to the residual GAT complexed to GAT-TsF.

To date all antigen-specific suppressor T-cell factors bear determinants encoded by the *H-2* gene complex (reviewed in 12). Comparison of those TsF that bear alloantigens encoded by the *I-J* region indicates variability in strain restrictions between donors and recipients. The discrepancy between requirements for syngenicity at I-J between GAT-TsF and carrier-specific TsF remains unresolved. Data presented here demonstrate that suppression of syngeneic and allogeneic PFC responses are mediated by the same molecules and that the association of GAT-TsF with a fragment of GAT does not mask inherent strain restrictions between donors and recipients of GAT-TsF. At least two significant differences remain between the GAT-TsF and carrier-specific TsF systems. First, carrier-specific TsF is analyzed by measuring suppression of hapten-specific responses stimulated by complexes of hapten and homologous carriers, whereas suppression by GAT-TsF is measured on PFC response to GAT itself. Second, the activity of GAT-TsF is analyzed on primary-immune responses whereas the activity of carrier-specific TsF is routinely analyzed on secondary PFC responses. Data from several other systems demonstrate that efficient collaboration between antigen-primed T cells and macrophages (Mϕ) or B cells requires that these cells are syngeneic at the *I* region of the major histocompatibility complex; whereas these restrictions do not appear to govern interactions between Mϕ and virgin T cells or B cells, (Reviewed in 13–16). By analogy, the discrepancy between requirements for syngenicity between donors and recipients of GAT-TsF and carrier-specific TsF may be a function of the immunological status of the recipient rather than a fundamental difference between the mediators. Experiments to resolve this issue are currently in progress.

Summary

The synthetic terpolymer of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT) fails to stimulate development of GAT-specific antibody responses in nonresponder mice but stimulates development of GAT-specific suppressor T cells that inhibit the development of normal anti-GAT plaque-forming cell responses to GAT complexed to methylated bovine serum albumin (MBSA). Extracts from lymphoid cells of GAT-primed but not control, nonresponder (DBA/1) mice contain a T-cell factor (GAT-TsF) that also specifically suppresses responses to GAT-MBSA by normal syngeneic spleen cells. The experiments reported in this communication demonstrate that: (*a*) extracts from all GAT-primed nonresponder mice tested contain GAT-TsF; (*b*) non-*H-2* genes do not restrict the production of GAT-TsF; (*c*) all nonresponder strains of mice regardless of their non-*H-2* genes are suppressed by GAT-TsF from all other strains bearing the nonresponder $H\text{-}2^{p,q,s}$ haplotypes; (*d*) suppression of GAT-MBSA responses by both syngeneic and allogeneic nonresponder spleen cells is mediated by a molecule encoded by the *H-2* gene complex; and (*e*) both syngeneic and allogeneic nonresponder mice are suppressed by purified GAT-TsF that lacks immunoreactive GAT.

It is a pleasure to thank Rita Allen and Isabel Diego, whose technical expertise contributed to the success of these experiments, and Sally Menkhus who skillfully typed this manuscript.

Received for publication 17 October 1977.

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NEUTRALIZATION OF MENINGOCOCCAL ENDOTOXIN BY ANTIBODY TO CORE GLYCOLIPID*

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The purpose of this study was to find an antibody that would neutralize the effects of meningococcal endotoxins from all capsular serogroups. We were interested in producing such an antibody because the capsular vaccines are serogroup-specific, and because there is no vaccine available for meningococcus (MGC)[1] B, Y, or other serogroups that may evolve into important pathogens. When our early unpublished experiments suggested that antibodies raised against the lipopolysaccharide (LPS) of one meningococcal serogroup would not be uniformly effective against the LPS of other serogroups, we turned to antibodies against *Escherichia coli* J5, the mutant of *E. coli* 0111 that is deficient in uridine 5'-diphosphate (UDP)-galactose epimerase. Because this bacterium cannot incorporate galactose into its LPS to make complete "0" side chains (1, 2), its core is accessible for stimulating antibody production to a wide range of bacteria with similar LPS cores.

Two separate lines of investigation from this laboratory encouraged us to test antibodies to this mutant *E. coli* against meningococcal endotoxemia. First, we found that purified meningococcal endotoxins were biochemically similar to enteric LPS, but were 10 times more potent for inducing the purpuric, necrotic lesions of the dermal Shwartzman phenomenon (3). This finding appeared to explain the high frequency of purpuric skin lesions in meningococcemia, and it provided a convenient way to test the effectiveness of antibodies to meningococcal LPS. Second, along with others in this laboratory, we showed that antiserum raised against *E. coli* J5 protected experimental animals against endotoxemia and bacteremia from such diverse bacteria as *E. coli*, encapsulated Klebsiella, and *Pseudomonas aeruginosa* (4–8). Finally, we reasoned that if J5 antibodies could counteract the effects of endotoxemia from diverse enteric bacilli, and if meningococcal and enteric LPS were similar both biochemically and biologically, antibodies to J5 might also neutralize meningococcal endotoxin.

Accordingly, we compared antibodies against *E. coli* J5 with antibodies against homologous and heterologous meningococcal serogroups for their capacity to prevent the dermal and general Shwartzman reactions induced by LPS from MGC A, B, and C. The superiority of antibodies to the *E. coli* mutant in these assays suggests that they will counteract the effects of meningococcal endotoxemia regardless of the capsular serogroup of the infecting strain.

Materials and Methods

Source of Microorganisms. Strains of *Neisseria meningitidis* from the collection at Walter Reed Army Institute of Research, Washington, D. C. were supplied by M. S. Artenstein. Strains

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* Supported by U. S. Army contract PATH/USA/DADA 17-72-C-2040.

[1] *Abbreviations used in this paper:* i.v., intravenous; LPS, lipopolysaccharide; MGC, meningococcus; PBS, phosphate-buffered saline; TSB, tripticase soy broth; UDP, uridine 5'-diphosphate.

A_1, B_{11}, and C_{11} were originally obtained from patients with meningitis or meningococcemia, and are the prototype strains used in vaccine studies. The letter in the designation of each strain of MGC indicates its serogroup; so that A_1 is serogroup A, B_{11} is serogroup B, and C_{11} is serogroup C. *E. coli* J5, a UDP-galactose epimeraseless mutant of *E. coli* 0111, was originally described by Elbein and Heath (1). Our strain differs in that it is no longer capable of incorporating galactose into the side chains of its LPS, even when galactose is present in the culture media (7).

MGC were stored in lyophiles, rehydrated with trypticase soy broth (TSB), and grown on blood agar overnight at 37°C under CO_2. *E. coli* J5 was stored on trypticase soy agar slants and subcultured onto blood agar for 24-48 h at 37°C.

Endotoxins. Endotoxin was extracted from each strain of MGC by our modification (3) of the phenol-water method of Westphal et al. (9). Briefly, this modification involved growing the cultures for 3 days, killing the cultures with a terminal concentration of 1% formaldehyde, discarding the middle layer between the phenol and water layers, and harvesting the endotoxin from the upper layer by ultracentrifugation instead of ethanol precipitation. After resuspension of the pellet, the material was lyophilized, collected, and weighed.

As previously described (3), each endotoxin was shown to contain less than 1% protein by the method of Folin and Ciocalteu (10), less than 1% RNA by optical density determinations at 280 and 260 nm, and to be free of detectable capsular contamination by the thiobarbituric acid technique of Warren (11) and by gas-liquid chromatography (2, 3). The presence of lipid A was confirmed by the method of Galanos et al. (12). All standards for chemical assays and gas-liquid chromatography were of the highest purity available from either Sigma Chemical Co., St. Louis, Mo., or Calbiochem, San Diego, Calif.

Immunization. Bacteria were grown in TSB under CO_2 for 48 h, harvested by centrifugation, washed three times in 0.15 M NaCl, and boiled for 2.5 h. After boiling, the cells were resuspended in 0.15 M NaCl and adjusted spectrophotometrically to a concentration of 5×10^9 cells/ml. 3-kg rabbits were exsanguinated 7 days after the last of six 1.0-ml intravenous (i.v.) injections of boiled cells given three times weekly for 2 wk. Nonimmune sera was obtained from nonimmune littermates.

Antibody Determinations. Hemagglutinating antibodies were measured in microtiter plates with human group 0 erythrocytes sensitized with the appropriate alkaline-treated endotoxin (13).

Separation and Purification of Immunoglobulins. 30- to 40-ml samples of pooled normal or immune rabbit serum were separated into 3 fractions on 5.0 × 100-cm glass columns packed with sterile Sephadex G-200 to a height of 90 cm. The eluant was phosphate-buffered saline (PBS) at pH 7.0, pumped upward from a reservoir by a peristaltic pump at 50-65 ml/h. 10-ml fractions were collected in an automatic fraction collector equipped with a 15-watt germicidal lamp. The optical density was recorded at 280 nm, and appropriate fractions were combined under sterile conditions. Chromatography of immunoglobulin classes was monitored by immunodiffusion against heavy-chain specific goat anti-rabbit IgM, IgA, and IgG. The frontal peak, which contained pure IgM by immunodiffusion, was restored to the original volume by concentration in dialysis tubing against polyethylene glycol at 4°C, and it was stored at 4°C for animal experiments. The second peak contained primarily IgG, but was contaminated with small amounts of IgA and IgM. After concentration, part of this material was stored at 4°C for animal experiments and part was further purified.

The 7S fraction was purified by chromatography over DEAE-Sephadex A-25 by the method of Hall et al. (14). 50- to 100-ml samples were dialyzed against buffer 1 and chromatographed on a 5 × 25-cm Pharmacia column (Pharmacia Inc., Piscataway, N.J.) packed with 75 g of sterile DEAE-Sephadex, also equilibrated in buffer 1. The protein peak eluted with buffer 1 (0.0175 M PBS at pH 6.3) was collected on the automatic fraction collector, pooled, and concentrated to a protein concentration equal to the original 7S peak (concentrated about 2 ×). This material was pure IgG by immunodiffusion and was retained for animal experiments. The second peak was eluted with 0.4 M PBS, pH 5.3. Before recharging the Sephadex by washing in buffer 1, it was treated with 1 M NaCl and 0.1 M NaOH.

Special precautions were taken to assure sterility as previously described (4). All sera and immunoglobulin fractions for prevention of the Shwartzman reactions were shown to be sterile and free of pyrogens.

Protection Against Meningococcal Dermal Necrosis (Local Shwartzman Phenomenon). Skin sites for the dermal Shwartzman reaction were prepared in groups of 10-20 1.0-1.5-kg rabbits by the intradermal injection of 1.75 μg in 0.25-ml volumes of MGC A, B, or C endotoxin. The reaction was provoked 21 h later by the i.v. injection of 0.5 ml of 1.25–1.5 μg of the corresponding endotoxin. Any hemorrhage or necrosis of the skin appearing 4–18 h after the provocative dose was recorded as a positive reaction. Experimental rabbits were given either 20 ml of antiserum or 15–20 ml of immune globulin fractions 19 h after the preparatory dose (2 h before the provocative dose). Control animals received the same volume of normal rabbit serum or nonimmune globulin adjusted to an equivalent protein concentration.

Protection Against Meningococcal Renal Cortical Necrosis (Generalized Shwartzman Phenomenon). Groups of 10–20 1.0–1.5-kg rabbits were given 20 ml of either normal or immune serum 3 days before they were prepared with 12.5–20 μg of MGC A, B, or C endotoxin i.v. in 0.5-ml volumes. Renal cortical necrosis was provoked 21 h later by the i.v. injection of 10–12.5 μg of the corresponding endotoxin. The animals were sacrificed 24 h after provocation, and their kidneys were judged positive if gross hemorrhage or necrosis were present on the external surface.

Statistical Methods. The chi-square test was used to determine whether or not there was a significant difference in the incidence of Shwartzman reactions between the groups.

Results

Production of Antiserum. Rabbits immunized with boiled cells of *E. coli* J5 uniformly caused hemagglutination of J5-sensitized erythrocytes to a titer > 1:256 by microtiter and > 1:1,000 by the tube technique. Immunization with *E. coli* J5 also caused a rise in hemagglutinins to meningococcal LPS generally equal to that obtained by immunization with the homologous MGC. Immunization with heterologous meningococcal boiled cells resulted in production of hemagglutinins, but the response was less uniform. The reciprocals of the hemagglutination titers are shown in Tables I, II, and III. Although protective antisera always contained hemagglutinins, there was no definite correlation between the height of the hemagglutinating antibody response and the degree of protection.

Prevention of Meningococcal Dermal Necrosis

MGC A ENDOTOXIN. Antiserum to *E. coli* J5, MGC A, MGC B, and MGC C all lowered the incidence of dermal necrosis significantly below that of animals which received nonimmune serum (87% positive; Table I). Although antisera to J5 and MGC A_I protected a larger percentage of animals (33 and 32% positive), they were not statistically more effective than antisera raised against MGC B_{11} and C_{11} (45 and 55% positive).

MGC B ENDOTOXIN. Only 28% of the rabbits given antiserum to J5 developed dermal necrosis, compared with 88% who received nonimmune serum ($P<0.0005$), and 59% who received heterologous meningococcal antiserum prepared against MGC A_I (Table II; $P=0.01$). Antiserum to MGC B_{11} (serogroup-homologous) was also protective, but not superior to MGC A_I antiserum. MGC C_{11} antiserum also protected all but 28% of the animals, but it was not superior to MGC A_I or B_{11} antiserum.

MGC C ENDOTOXIN. J5 antiserum protected the largest percentage of rabbits in this experiment and was superior to nonimmune and anti-meningococcal A_I sera (Table III). Antisera against MGC B_{11} and C_{11} were also protective.

COMPOSITE RESULTS. The results of the experiments shown in Tables I, II, and III are combined in Table IV. In this table the results of protection experiments with each antiserum against all three meningococcal endotoxins are combined, so that the protective capacity of each meningococcal antiserum

TABLE I

*Prevention of Dermal Shwartzman from MGC A_1 Endotoxin with E. coli J5 and Meningococcal Antisera**

Serum	Hemagglutination titer‡	Number and percent positive	*P* value§
Nonimmune	0	52/60 (87)	–
Anti-MGC A_1	128	12/38 (32)	<0.0005
Anti-*E. coli* J5	32	10/30 (33)	<0.0005
Anti-MGC B_{11}	8	9/20 (45)	<0.0005
Anti-MGC C_{11}	8	11/20 (55)	<0.004

* The skin of 1-1.5-kg white New Zealand rabbits was prepared with an intradermal injection of 1.75 μg of MGC A endotoxin in a 0.25-ml volume. 19 h later, 20 ml of serum was given in one lateral ear vein, 2 h before the provocative i.v. dose of 1.25 μg of MGC A endotoxin in a 0.5-ml volume was given in the other ear. Skin sites were examined at 4 and 24 h, and judged positive if hemorrhage or necrosis of the skin occurred within 24 h of the provocative dose.

‡ Reciprocal of hemagglutination titer against human group O erythrocytes sensitized with alkaline-treated MGC A_1 endotoxin.

§ *P* values are in comparison to the normal serum control and were calculated by the chi-square technique.

TABLE II

*Prevention of Dermal Shwartzman from MGC B_{11} Endotoxin with E. coli J5 and Meningococcal Antisera**

Serum	Hemagglutination titer‡	Number and percent positive	*P* value
Nonimmune	0	52/59 (88)	–
Anti-MGC B_{11}	32	14/30 (47)	<0.0005
Anti-*E. coli* J5	64	11/40 (28)	<0.0005§
Anti-MGC A_1	32	17/29 (59)	<0.002§
Anti-MGC C_{11}	16	5/18 (28)	<0.0005

* Experiments were conducted as stated in the text and in Table I except that B_{11} endotoxin was used to prepare (1.75 μg) and provoke (1.5 μg) the dermal Shwartzman reactions.

‡ Reciprocal of hemagglutination titer against human group O erythrocytes sensitized with alkaline-treated MGC B_{11} endotoxin.

§ Protection by *E. coli* J5 antiserum (28% positive) was superior to that from MGC A_1 antiserum (59% positive), $P<0.01$.

is examined against heterologous as well as homologous LPS. These data show that antiserum to *E. coli* J5 was more protective (33% positive) than nonimmune serum (87% positive; $P<0.0005$), MGC A antiserum (51% positive; $P<0.015$), MGC B antiserum (49% positive, $P<0.03$), and MGC C antiserum (47% positive; $P<0.06$).

Table V compares the results of all protection experiments with *E. coli* J5 antiserum to each meningococcal antiserum tested only against the serogroup-heterologous endotoxins. In this analysis which represents a more realistic comparison of the cross-reactivity of antiserum and LPS, J5 antisera protected

TABLE III

*Prevention of Dermal Shwartzman from MGC C_{11} Endotoxin with E. coli J5 and Meningococcal Antisera**

Serum	Hemagglutination titer‡	Number and percent positive	*P* value
Nonimmune	0	35/40 (88)	–
Anti-MGC C_{11}	32	16/30 (53)	<0.002
Anti-*E. coli* J5	64	12/30 (40)	<0.0005§
Anti-MGC B_{11}	16	11/19 (58)	<0.011
Anti-MGC A_1	32	15/20 (75)	No protection§

* Experiments were conducted as stated in the text and in Table I except that C_{11} endotoxin was used to prepare (1.75 μg) and provoke (1.5 μg) the dermal Shwartzman reactions.

‡ Reciprocal of hemagglutination titer against human group O erythrocytes sensitized with alkaline-treated MGC C_{11} endotoxin.

§ Protection by *E. coli* J5 antiserum (40% positive) was superior to that from MGC A_1 antiserum (75% positive), $P<0.015$.

TABLE IV

*Prevention of Dermal Shwartzman from MGC Endotoxins – Superiority of E. coli J5 over MGC Antisera**

Serum	Number and percent positive	*P* value‡
Nonimmune	139/159 (87)	<0.0005
Anti-*E. coli* J5	33/100 (33)	–
Anti-MGC A_1	44/87 (51)	<0.015
Anti-MGC B_{11}	34/69 (49)	<0.03
Anti-MGC C_{11}	32/68 (47)	<0.06

* The results of protection experiments with each serum against endotoxins of MGC A_1, B_{11}, and C_{11} are combined in this table.

‡ *P* values compare each serum to the results obtained with *E. coli* J5 antiserum.

a higher percentage of rabbits (33% positive) than did heterologous meningococcal antisera (45% positive; $P<0.002$).

Prevention of Meningococcal Renal Cortical Necrosis. E. coli J5 antiserum also prevented the renal cortical necrosis of the generalized Shwartzman reaction, regardless of the serogroup of meningococcal endotoxin. Tables VI, VII, and VIII show that the protection afforded by J5 antiserum against renal cortical necrosis from MGC A_1 (18% positive), MGC B_{11} (30% positive), and MGC C_{11} (22% positive) was significant and at least as effective as that provided by homologous antisera. Heterologous meningococcal antisera did not prevent renal cortical necrosis induced by LPS from MGC B_{11} and C_{11} (Tables VII and VIII).

Protection with J5 Immunoglobulin Fractions

PURITY. The 19S fractions of whole rabbit serum chromatographed over Sephadex G-200 contained only IgM by immunodiffusion and were used in protection experiments without further purification. Because the 7S fractions of

TABLE V

*Prevention of Dermal Shwartzman from Meningococcal Endotoxins: Superiority of E. coli J5 over Heterologous Meningococcal Antisera**

Serum	Number and percent positive
All heterologous MGC antisera	68/125 (45)‡
All *E. coli* J5 antisera	33/100 (33)‡

* The results of protection experiments with each serum against only the serogroup-heterologous endotoxins are combined in this table. Thus, the results of J5 antiserum against all three meningococcal endotoxins are compared with the results of meningococcal antisera tested only against the endotoxins from the two serogroup-heterologous MGC.

‡ *E. coli* J5 antiserum is superior to heterologous MGC antisera ($P<0.002$).

TABLE VI

*Prevention of Renal Cortical Necrosis from MGC A_1 Endotoxin with E. coli J5 and Meningococcal Antisera**

Serum	Number and percent positive	*P* value‡
Nonimmune	15/36 (42)	–
Anti-MGC A	7/39 (18)	0.025
Anti-*E. coli* J5	4/39 (10)	0.0025

* Rabbits received 20 ml of serum 3 days before they were prepared with 20 μg of i.v. endotoxin. The reaction was provoked 21 h later with 12.5 μg of i.v. endotoxin. The animals were sacrificed 24 h after provocation and kidneys were judged positive if gross hemorrhage and necrosis were present on the external surface.

‡ *P* values are in comparison to the normal serum control and were calculated by the chi-square technique.

TABLE VII

*Prevention of Renal Cortical Necrosis from MGC B_{11} Endotoxin with E. coli J5 and Meningococcal Antisera**

Serum	Number and percent positive	*P* value
Nonimmune	7/8 (88)	–
Anti-MGC B_{11}	3/8 (38)	<0.15
Anti-*E. coli* J5	3/10 (30)	<0.05
Anti-MGC C_{11}	6/10 (60)	No protection

* Experiments were conducted as stated in the text and in Table VI except that B_{11} endotoxin was used to prepare (12.5 μg) and provoke (10 μg) renal cortical necrosis.

both nonimmune and immune sera showed contaminating IgA, portions of each were further purified over DEAE-Sephadex A-25. The first peak eluted from the DEAE-Sephadex contained only IgG by immunodiffusion and was stored at 4°C for protection studies.

TABLE VIII

*Prevention of Renal Cortical Necrosis from MGC C_{11} Endotoxin with E. coli J5 and Meningococcal Antisera**

Serum	Number and percent positive	*P* value
Nonimmune	11/16 (69)	–
Anti-MGC C_{11}	7/19 (37)	0.06
Anti-*E. coli* J5	4/18 (22)	0.006
Anti-MGC A_1	5/10 (50)	No protection

* Experiments conducted as stated in the text and in Table VI except that C_{11} endotoxin was used to prepare (15 μg) and provoke (10 μg) renal cortical necrosis.

TABLE IX

*Prevention of Dermal Shwartzman from MGC A_1 Endotoxin with Partially Purified Immunoglobulins of E. coli J5 Antiserum**

Globulin	Number and percent positive	*P* value
Nonimmune 19S	16/19 (84)	–
Nonimmune 7S	14/20 (70)	–‡
E. coli J5 19S	14/20 (70)	No protection
E. coli J5 7S	7/19 (37)	<0.05‡

* Experiments were conducted as stated in the text and in Table I except that 15 ml of 7S or 19S immunoglobulin collected over Sephadex G-200 was tested instead of 20 ml of whole serum.

‡ *E. coli* 7S globulin was superior to nonimmune 7S globulin $P<0.05$. Immune 19S was not protective.

TABLE X

*Prevention of Dermal Shwartzman from MGC A_1 Endotoxin with Purified Immunoglobulin G of E. coli J5 Antiserum**

Serum or globulin	Number and percent positive	*P* value
Nonimmune IgG	9/10 (90)	–
E. coli J5 Serum	2/10 (20)	<0.003
E. coli J5 IgG	1/10 (10)	<0.0005

* Experiments were conducted as stated in the text and in Table I except that the 7S fractions of Sephadex G-200 were further purified over DEAE Sephadex A-25 and concentrated to protein concentrations approximately equivalent to that of the Sephadex G-200 7S fractions. Each rabbit was given 20 ml of either whole J5 serum or purified IgG 2 h before the provocative dose of endotoxin.

The 19S and 7S fractions from Sephadex G-200 were restored to the original volume of the serum before they were used in animal experiments. Normal and J5 immune IgG purified over DEAE were concentrated twofold to approximately the total protein content of the original 7S Sephadex fractions, before they were used in animal experiments.

PREVENTION OF DERMAL NECROSIS WITH IMMUNOGLOBULIN FRACTIONS. The results of protection experiments with nonimmune and J5 immune 7S and 19S fractions from Sephadex G-200 are shown in Table IX. Immune 7S was protective ($P<0.05$) whether compared to nonimmune 7S, nonimmune 19S, or immune 19S. Immune 19S was not protective.

The next experiment shown in Table X compared purified nonimmune and J5 immune IgG from DEAE Sephadex with *E. coli* J5 antiserum. Immune IgG (10% positive) was as protective ($P<0.005$) as whole *E. coli* J5 antiserum (20% positive; $P<0.003$) when compared to nonimmune IgG (90% animals positive).

Discussion

We have shown in these studies that antibodies to *E. coli* J5, a rough mutant of *E. coli* 0111, can protect against the two most dramatic manifestations of meningococcal endotoxemia: dermal purpura and renal cortical necrosis. Because this mutant is deficient in UDP-galactose epimerase, it cannot build the complex "0" antigenic side chains that mask the LPS core of most endotoxins, including *E. coli* 0111 and MGC A_1, B_{11}, and C_{11}. This enzymatic block, which is equivalent to that of the Rc forms of Salmonella (15), exposes the core so that it can stimulate antibodies capable of cross-reactions with all antigenically similar endotoxin cores. It is this structural property of *E. coli* J5 that is responsible for stimulating antibodies that protect against endotoxemia and bacteremia due to *E. coli* (4–7), Salmonella (4–6), Klebsiella (7), *P. aeruginosa* (8), and meningococcal LPS, regardless of serogroup. We have shown previously that the "0" antigenic side chains, responsible for serogrouping enteric bacilli, interfere with antibody production to the antigenically similar LPS core. In these studies, antibodies to the parental form of J5, *E. coli* 0111, unlike J5 antibodies, would not protect against diverse gram-negative bacilli such as Klebsiella (7) and *P. aeruginosa* (8).

The current study with meningococcal LPS shows that the oligosaccharide side chains of meningococcal LPS also interfere with serogroup heterologous protection. Whereas antibodies to *E. coli* J5 protected against dermal and renal necrosis induced by LPS from MGC A_1, B_{11}, and C_{11}, antibodies to MGC A_1 failed to protect against dermal necrosis or renal cortical necrosis induced by MGC C_{11} LPS (Tables III and VIII). Similarly, MGC C_{11} failed to protect against renal cortical necrosis induced by MGC B_{11} (Table VII). Although antiserum to MGC A_1 prevented dermal necrosis from B_{11} LPS, this protection was inferior to that from J5 antibodies (Table II). Finally, the protection by J5 antibodies against dermal necrosis induced by LPS from all three meningococcal serogroups was better than anti-meningococcal sera, even when the homologous endotoxin was included in the calculations (Table IV). The complete absence of cross protection in some experiments shows that meningococcal endotoxins are antigenically different. Because antibodies to the common core of LPS (*E. coli* J5) are completely cross-reactive, this antigenic variability must reside elsewhere in the LPS complex, most likely in the "0" antigenic units. Antigenic variability of "0" side chains is well known among enteric bacilli, but has not been generally appreciated in meningococcal LPS, although Zollinger et al. (16) showed incomplete serological cross-reactivity of antisera raised against MGC B and C.

The experiments with immunoglobulin fractions (Table IX and X) indicate that the protective factor in J5 antiserum is antibody. These studies, which show that purified IgG is as effective as whole antiserum, are important because a satisfactory in vitro assay of cross-reactivity between J5 antiserum and meningococcal endotoxins is not yet available. Our hemagglutinin results indicate that cross-reactive antibodies were stimulated by immunization with J5, but there was no definite correlation between the height of antibody response and the degree of protection. More promising results have been obtained with the solid-phase radioimmunoassay. Sadoff et al. (17), have shown that J5 antibodies react strongly with all MGC tested in this assay.

The failure of isolated IgM to prevent dermal necrosis is puzzling. It is possible that IgM reacts with whole bacteria rather than purified LPS, or that IgM antibodies can react only with certain endotoxins. In previous studies of immunoglobulin fractions directed against the homologous endotoxin, both anti-*E. coli* 0111 7S and 19S fractions were protective against renal cortical necrosis induced by 0111 endotoxin (6). Some antibodies of the IgM class should be present at 21 days when immunized rabbits were exsanguinated, but it is possible that the concentration of protective antibodies is too low to detect in animal assays.

The demonstration that purified antibodies raised against an *E. coli* mutant can prevent the manifestations of meningococcal endotoxemia suggests that the core of meningococcal endotoxin is the toxic moiety, and underscores the antigenic similarity of LPS from otherwise unrelated bacteria. Moreover, this antibody may provide an additional immunological weapon against meningococcemia. The pioneering work of Goldschneider et al. (18) and Gotschlich et al. (19, 20) at Walter Reed has provided capsular vaccines that prevent meningococcal disease caused by MGC of groups A and C (21–23). Unfortunately, this protection is serogroup-specific. Group B capsule is not immunogenic in man (24), and there is no vaccine available for serogroup Y or other groups that may evolve into important pathogens. Because of the serogroup specificity of the capsular vaccines, the nonimmunogenicity of group B capsule, and the pathogenic potential of other meningococcal serogroups, investigators have turned to studies of outer membrane antigens that might cross-react between capsular groups (16, 25–27). These studies have resulted in the identification of a number of different outer membrane serotypes that cross serogroup lines. The protective capacity of serotype antibodies against group B strains tested in chick embryos, however, appears to be serogroup-specific (28); in other words, antisera raised against whole MGC B of different serotypes showed primarily group-specific protection. Serogroup and serotype antibodies did appear to provide synergistic protection against the homologous serogroup.

On the other hand, antiserum raised against *E. coli* J5 protects rabbits from meningococcal endotoxemia regardless of serogroup. Similarly, gamma globulin prepared from these broad-spectrum antibodies should counteract the devastating effects of meningococcal endotoxemia in patients infected with any capsular serogroup.

Summary

Antibodies to *Escherichia coli* J5, a uridine 5′-diphosphate-galactose epimeraseless mutant of *E. coli* 0111, neutralized meningococcal endotoxemia from all

three major capsular serogroups. We chose the dermal necrosis of the local Shwartzman phenomenon and the renal cortical necrosis of the general Shwartzman phenomenon as assays because these are the hallmarks of meningococcemia, and because meningococcal lipopolysaccharide (LPS) is a uniquely potent cause of dermal purpura and necrosis. Meningococcal antisera raised against LPS from MGC A, B, and C also provided good protection against endotoxemia from the homologous capsular groups, but it was inconsistent against the heterologous serogroups. The superiority of J5 antibodies (purified IgG as well as antiserum) is probably due to the fact that J5 LPS contains only the endotoxin core. Consequently, immunization with this mutant stimulates production of antibodies to core LPS without interference by the "0" antigenic determinants of the side chains.

These observations indicate that the endotoxin core is the toxic moiety of meningococcal LPS, that the core LPS of meningococcus (MGC) is immunologically similar to enteric LPS, and that the antigenically variable "0" side chains of MGC LPS interfere with antibody production against the common core. They also suggest that antibodies prepared against this *E. coli* mutant could interrupt the devastating course of meningococcal endotoxemia in man, regardless of the capsular serogroup of the infecting strain.

Received for publication 30 November 1977.

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LYMPHOCYTES TRANSFORMED BY EPSTEIN-BARR VIRUS

Induction of Nuclear Antigen Reactive with Antibody in Rheumatoid Arthritis*

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Rheumatoid arthritis (RA)[1] is a disease in which immune mechanisms have been considered to play a significant if not the major role in pathogenesis (1–3). There have been many studies which support this hypothesis. The synovial tissue of the peripheral joints is the most important site of tissue injury, and the inflammatory cell infiltrate is composed mostly of lymphocytes and plasma cells with a striking absence of polymorphonuclear leukocytes (4–6). Immunofluorescent studies have shown abundant amounts of immunoglobulin and complement in synovial tissue (7, 8), and synovial fluids contain aggregates of immunoglobulin, rheumatoid factor-gamma globulin complexes, and nuclear antigen-antibody complexes (9). The etiology of RA has been considered by many investigators to be due to the presence of some specific and persistent antigenic stimulation. Much work has been directed towards searching for serum antibodies to bacteria or viruses in the hope that there might be clues pointing towards a specific microbial agent. The results have been conflicting and generally inconclusive (10, 11).

Recently, we demonstrated that the sera of patients with RA contained an antibody which reacted with a cell-associated antigen present in human lymphocyte cell lines. This was demonstrated initially by precipitin lines in immunodiffusion between rheumatoid sera and soluble extracts of tissue culture cells, and the antibody in rheumatoid sera was referred to as RA precipitin (RAP) (12, 13). Subsequently, the antigen was demonstrated by immunofluorescence (14) and it has now been shown to be distributed predominantly in the nucleus of tissue culture B-lymphocyte cell lines. Our hypothesis was that this

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* Publication number 1277 from the Immunology Departments, Scripps Clinic and Research Foundation. This work was supported by National Institutes of Health grants AM 21356 and CP 43375 and Nol-CO-25423.

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[1] *Abbreviations used in this paper:* EB, Epstein-Barr; EBNA, Epstein-Barr nuclear antigen; EBV, Epstein-Barr virus; FCS, fetal calf serum; HCL, human cell line; RA, rheumatoid arthritis; RANA, RA-associated nuclear antigen; RAP, RA precipitin.

antibody could be directed against a neoantigen in transformed lymphocytes. In this study, we present evidence to show that the nuclear antigen reactive with antibody in the sera of patients with RA could be induced in lymphocytes transformed by Epstein-Barr (EB) virus. The nuclear antigen appears to be different from the Epstein-Barr nuclear antigen (EBNA).

Materials and Methods

Transformation of Human Peripheral Blood Lymphocytes with EB Virus. 200 ml of blood from each of three normal individuals was collected in heparin, and the leukocytes separated by dextran sedimentation. 30 ml of leukocyte-rich plasma was added to 10 ml of leukocyte separation medium (Bionetics Laboratory Products, Kensington, Md.), centrifuged at 2,000 rpm at room temperature for 10 min, and the sedimented leukocytes washed with RPMI 1640 (Associated Biomedic Systems, Inc., Buffalo, N.Y.). After centrifuging at 1,500 rpm for 10 min, the procedure was repeated a second time. The method usually yielded 40 ml containing 1×10^6-1.5×10^6 leukocytes/ml.

B-lymphocyte line (B95-8), (supplied by Dr. Berge Hampar, National Cancer Institute) producing Epstein-Barr virus (EBV) was used as the source of virus for transformation. B95-8 cells were cultured in RPMI 1640 with 10% fetal calf serum supplemented with glutamine and nonessential amino acids (Flow Laboratories, Inc., Rockville, Md.) and allowed to reach a peak growth of 2.5×10^6 cells/ml. The cells were spun down, resuspended at 2×10^7 per ml in RPMI 1640, and sonicated at 4°C. The sonicate was then centrifuged and the supernate filtered through 0.45 μm Millipore filter (Millipore Corp., Bedford, Mass.). The virus pool was divided into small portions and stored at −70°C to be used as needed.

20 ml of leukocyte suspension containing 2×10^6 cells/ml was sedimented by centrifugation and resuspended in 1 ml of RPMI 1640 containing fetal calf serum (FCS). The cells were added to 1 ml of undiluted virus from B95-8 cells, and the suspension left at 37°C for 30 min to allow adsorption of EBV. Infected cells were pipetted into two plastic 250-ml tissue culture flasks (Lux Scientific Corp., Newbury Park, Calif.), and 15 ml of fresh media containing FCS and supplements were added. Control cells which were not exposed to virus were handled separately and cultured at the same concentrations. Cultures were incubated as stationary suspension cultures at 37°C in a humidified atmosphere containing 5% CO_2. The cell cultures were frequently examined for cell morphology and given fresh media every 7 days. All three preparations of cells infected with EB virus were noted to undergo changes in cell culture characteristic of virus-induced transformation. By day 7, some large lymphocytes were forming clusters on the bottom of the flask. By day 14, these clusters of lymphocytes were greatly enlarged, and in addition, clumps of cells were present in suspension in the culture medium. From day 20 to 23, the number of large lymphocytes in suspension had increased significantly and the cells could be transferred for subculture on a rotary shaker. In contrast, two uninfected control cell lines showed decreasing numbers of cells on days 20–23 and in subsequent weeks, could not be sustained in culture. Cell cultures were harvested at various times and prepared for immunofluorescent studies by cytocentrifugation on to glass slides.

Extraction of Soluble Antigen from Cells. Suspensions of cells (between 500 and 1,000 ml) from each cell line at 1×10^6 per ml were centrifuged and extracted by the same method described previously for WiL_2 cells (15). Briefly, this consisted of recovering cells by low speed centrifugation and sonication of isolated cells in a solution of 0.25 M sucrose and 0.04 M calcium chloride buffered at pH 6.2 with 0.01 M phosphate. After centrifugation at 105,000 *g* for 30 min, the supernate of sonicated cells was used as the cell extract for studies in immunodiffusion in agarose against RA sera.

Immunological Studies. Two immunological assay systems were used for detection of antigen which reacted with serum antibody in RA sera (14). Three prototype sera from patients with RA had been previously characterized and were used throughout these studies. These sera reacted with soluble antigens in extracts of WiL_2 lymphocytes by immunodiffusion to give precipitin lines which were immunologically different from precipitin lines caused by antibodies to nuclear antigens such as DNA, nucleoprotein, Sm, and ribonucleoprotein previously described (16). It was also previously demonstrated that the precipitin reaction was not caused by rheumatoid factor (14). In immunodiffusion studies reported here, extracts from EBV-transformed cell lines were placed in wells adjacent to wells containing WiL_2 extracts, and tested against prototype RA sera. Precipitin lines with extracts of transformed cells showing complete fusion with WiL_2 lines were taken to demonstrate immunological identity of antigens in the transformed cell line extracts.

Immunofluorescence was the second assay system used. The three prototype RA sera were used as the intermediate reagents in the indirect immunofluorescent test with fluorescein-conjugated anti-human IgG as the fluorescent marker. These three RA sera were negative for nuclear staining when tissue sections from organs (mouse or human kidney and liver) were used as substrates but reacted with WiL_2 cells to give nuclear staining of discrete finely speckled character. The specificity of the reaction had been previously demonstrated (14). It had also been shown previously that nuclear staining was most clearly demonstrated when cells were fixed with dry heat at 37°C for 30 min. Other fixatives such as acetone and alcohol resulted in loss of reactivity of this nuclear antigen. The different cell lines used in this study were prepared for immunofluorescence by washing the harvested cells in RPMI 1640 medium. Flat preparations of cells were made by cytocentrifugation on to glass slides and heated at 37°C for 30 min. They were reacted with RA sera for 30 min at room temperature, washed, and reacted with fluorescent anti-human IgG. RA sera were usually used at 1:4 dilution. Several sera from patients with Burkitt's lymphoma were made available for these studies through the courtesy of Dr. Guy de Thè, World Health Organization, Lyon, France.

Differentiation from EBNA. Reedman and Klein (15) have described an EBNA in EBV-infected cells, and it was important to determine whether the nuclear antigen reacting with RA sera was similar or different. For determination of EBNA, cell lines were fixed in chilled acetone as described (15). Reference sera containing antibodies to EBNA and sera negative for EBNA antibodies (the latter also used as a source of complement) were supplied by Dr. B. Hampar. Nuclear staining characteristic of EBNA was compared to that given by RA sera.

Other Human and Simian Cell Lines Infected with EBV and Related Herpes Virus. Several cell lines of human and simian origin (cotton-topped marmoset, orangutan, baboon, and owl monkey) were obtained from the Frederick Cancer Research Center, Frederick, Md. (17). The human cell lines were umbilical cord lymphocytes, transformed with EBV (HCL-3/B95-8) and herpes virus papio (HCL-3/KMPG-1). Two simian cell lines CP-81 (18) and 594-S (19) were lymphocyte lines established from animals who developed spontaneous lymphoid disease. Three other simian lymphocyte cell lines were from animals who developed lymphoid disease after in vivo inoculation with herpes virus (531-H, 1605-S, and MLC-1 [20]) and another line (B95-8) was derived from in vitro transformation of isolated lymphocytes. With the exception of WiL_2, all the cell lines were under study in the laboratory of one of the authors (H. Rabin) and were being characterized with respect to the transforming activity and antigenicity of the herpes viruses present in simian cells (17). The cells were prepared for immunofluorescent studies as described above and reacted with RA and serum with EBNA antibody to determine the presence or absence of RA-associated nuclear antigen (RANA) and EBNA, respectively. Immunodiffusion studies were not performed with these cell lines.

Results

Immunofluorescent Studies on Cultured Human Lymphocyte Cell Lines. The indirect immunofluorescent test was used to detect the presence of antigen reactive with antibodies in RA sera. The characteristic pattern of nuclear staining seen in WiL_2 cells is illustrated in Fig. 1 a. In this cell line, which is known to contain EB viral genome, the pattern of nuclear staining by using RA sera and fluorescein-conjugated anti-IgG reagent is demonstrated as distinct fine speckled nuclear staining. This pattern of nuclear staining is unique and can be readily distinguished from nuclear staining produced by antibodies to DNA, histones, or nonhistone proteins such as Sm antigen and ribonucleoprotein. In the human lymphocytes infected with EBV, no staining could be detected in cells taken at day 1 and day 14 of culture. By day 20, nuclear staining began to appear in the EBV-infected cells, showing up as a few distinct fine speckles of nuclear staining, as illustrated in Fig. 1 b. This was present in

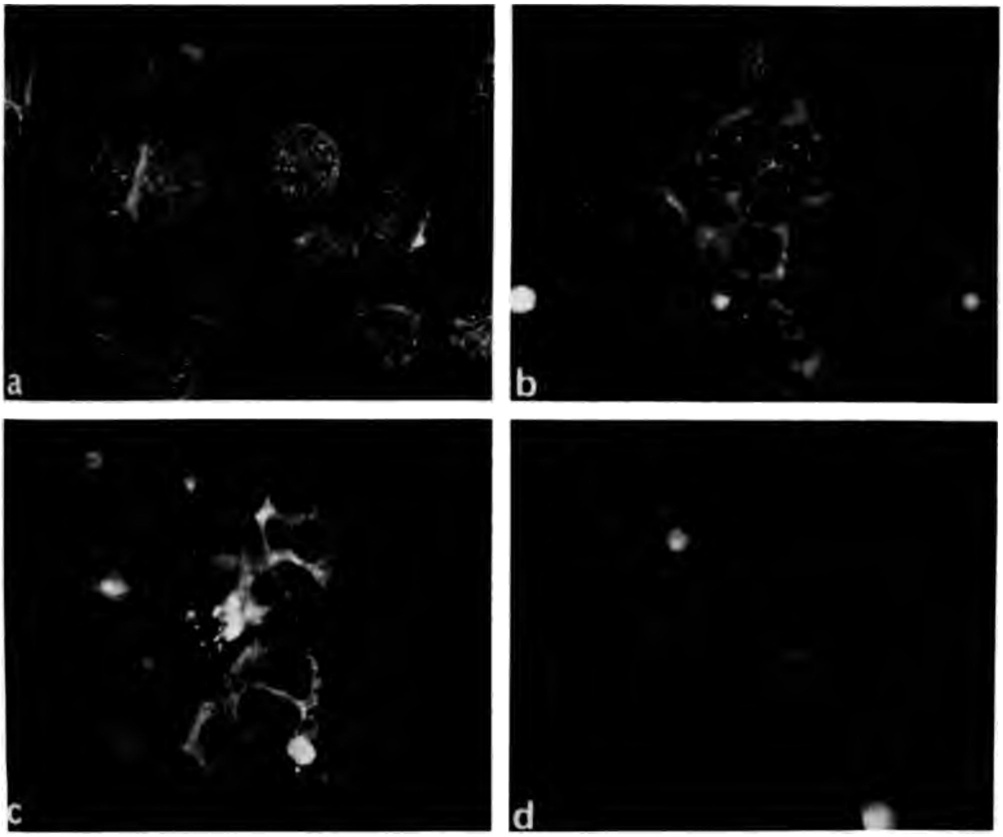

FIG. 1. Indirect immunofluorescence to demonstrate the presence of nuclear antigen reactive with antibody in RA sera. Tissue culture cells were cytocentrifuged on to glass slides, reacted with RA serum containing antibody, and stained with fluorescein-conjugated anti-human IgG. (a) demonstrates the discretely distributed finely speckled nuclear staining observed on WiL_2 cells; (b) was EBV-infected peripheral blood leukocytes at day 20 of culture. A few discretely distributed fine nuclear speckles were seen at this time and became more numerous after the cells became more transformed into continuous lines; (c) were the same EBV-infected cells (day 20) reacted with normal human serum; and (d) was a control noninfected cell culture at day 20, reacted with the same RA serum as in (b), showing absence of nuclear staining.

approximately 10–20% of the infected cells. By day 49, at least 70% of the cells were staining in a pattern similar to the staining seen in WiL_2 cells shown in Fig. 1 a. Fig. 1 c demonstrates that the same EBV-infected cells (at day 20) did not react with normal human serum, and Fig. 1 d shows that the control uninfected cell culture did not show nuclear staining with RA sera. Two other infected cell lines showed similar induction of this nuclear antigen and the corresponding control, uninfected cell cultures were completely negative.

EBV-infected cells at day 49 were extracted as described, and the soluble extract studied in immunodiffusion tests as illustrated in Fig. 2. A RA serum (RAP) containing precipitating antibody to WiL_2 extract also showed precipitin lines with D.J. and K.H. extracts (two EBV-infected cell preparations), and these lines fused in complete immunological identity with the WiL_2 precipitin line. It

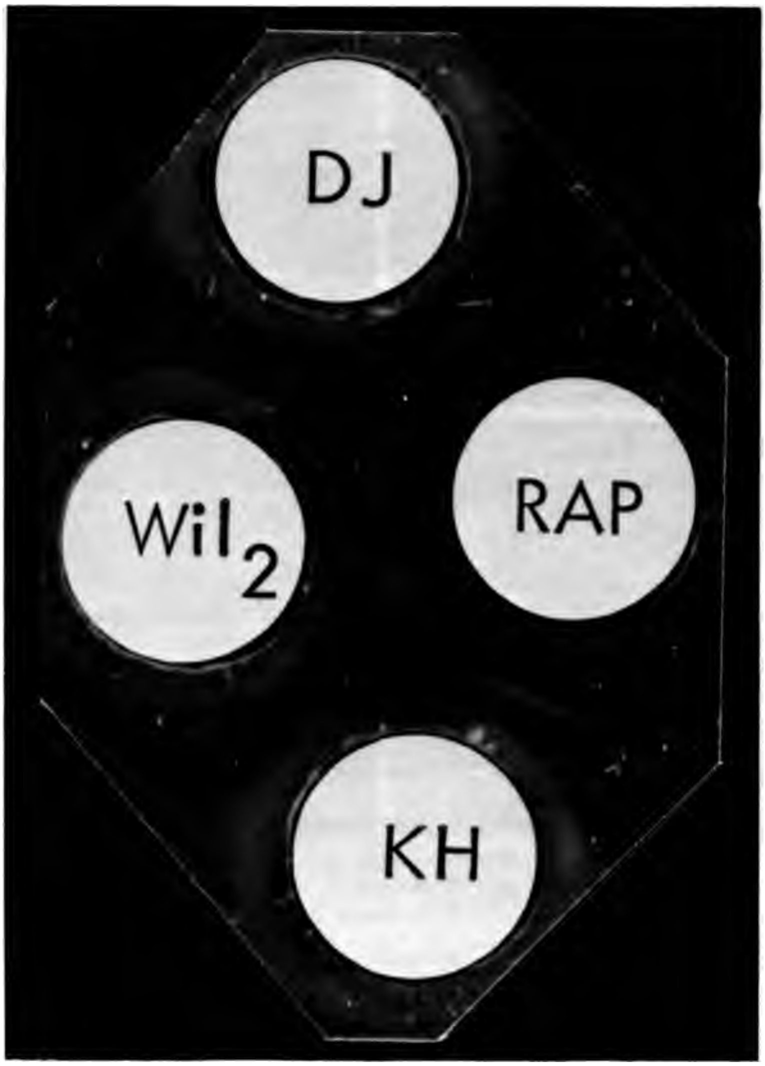

FIG. 2. Immunodiffusion study between RA serum containing precipitating antibody (RAP) and cell culture extracts. D.J. and K.H. were human peripheral blood leukocytes infected with EB virus and cell cultures harvested at day 49. Soluble antigens were extracted in the same manner as that used for the lymphocyte line WiL_2. Precipitin lines developed between RAP, and the three cell culture extracts and the precipitin lines fused completely with one another to show immunological identity.

was further shown that when RAP serum was absorbed with WiL_2 extract to remove precipitating antibody, the absorbed serum was no longer reactive in immunodiffusion with D.J. and K.H. extracts.

Studies on Human and Simian Lines Containing EBV and Related Herpes Viruses. The above studies suggested that the nuclear antigen reactive with serum antibody in RA sera could be induced in human lymphocytes by EBV. The availability of a number of well-characterized human and simian lymphocyte cell lines provided the opportunity to determine whether the nuclear

TABLE I
EBNA and RANA in Human and Simian Cell Lines

ine	Animal	Transforming virus	Lymphocyte cell type	Immunofluorescent staining for	
				EBNA	RANA
	Human spleen	EBV	B	+++	+++
8	Human umbilicus	EBV	B	+++	++
PG-1	Human umbilicus	*Herpes* (H) *papio*	B	−	−
	Owl monkey-in vitro EBV-induced lymphoreticular hyperplasia	EBV	B	+++	+
	Cotton-topped marmoset-in vitro EBV-transformed lymphocytes	EBV	B	+++	+
	Cotton-topped marmoset-in vivo EBV-induced lymphoma	EBV	B	++	+
	Orangutan with monomyelogenous leukemia	*Herpes pongo*	Undifferentiated	+	−
	Baboon with lymphoreticular hyperplasia	*Herpes papio*	B	−	−
	Cotton-topped marmoset-*H. saimiri* induced lymphoma	*Herpes saimiri*	T	−	−

gen was induced only by EBV or was also inducible by related herpes ses of simian origin. It can be noted (Table I) that the human cord hocyte line infected with EBV (HCL-3/B95-8) was positive for EBNA and he nuclear antigen reactive with RA sera, but cord cells infected with es virus papio were negative for both antigens. We observed that simian cell which had been transformed in vitro with EBV (B95-8) or obtained from als inoculated in vivo with EBV (531-H and 1605-S) also contained the ear antigen reactive with RA sera. On the other hand, simian cell lines ying herpes-like viruses such as herpesvirus pongo (CP-81, of orangutan n), herpesvirus papio (594-S, of baboon origin), and herpesvirus saimiri C-1, of squirrel monkey origin) were negative for the nuclear antigen tive with RA sera. It was noted that the WiL$_2$ cell line was stronger in unofluorescence for the RA-associated nuclear antigen than other cell lines ive for this antigen. The cell line MLC-1, which was from a marmoset h developed lymphoma after inoculation with herpesvirus saimiri had been acterized as a T lymphocyte and was negative for either EBNA-like or the associated nuclear antigen. These studies showed a close relationship een RA-associated nuclear antigen and EBV-infected cells, but lack of a ionship with cells infected with herpes-related viruses of simian origin. ough most cells containing RA-associated nuclear antigen also contained IA, there was lack of complete concordance, as shown by presence of EBNA-antigen in cell line CP-81 but absence of RA-associated nuclear antigen.

fferentiation from EBNA. A serum containing antibody to EBNA (from B. Hampar) and negative for precipitating antibody to WiL$_2$ extract was pared with an RA serum negative for antibody to EBNA but positive for ipitating antibody to WiL$_2$. WiL$_2$ cells which have both EBNA and the RA-ciated nuclear antigen were used as substrate in indirect immunofluores-e. Staining for RA nuclear antigen by RA serum was best demonstrated anti-IgG reagent and was not amplified with anti-C3. In contrast, staining EBNA by anti-EBNA serum was not demonstrable with anti-IgG reagent was strongly positive with anti-C3, as described by Reedman and Klein

TABLE II

Lack of Relationship between Antibodies to RANA and EBNA

Sera	Immunofluorescent staining for		Precipitating antibody to RANA
	RANA	EBNA	
	RA		
VM	+ (32)	+ (256)	+ (16)
VI	+ (16)	–	+ (4)
TF	+ (16)	+ (512)	+ (4)
	Burkitt lymphoma		
1440	–	+ (5,120)*	–
1020	–	+ (2,560)	–
1023	–	+ (80)	–
1053	–	+ (1,280)	–
1003	–	–	–
	Normal sera		
CP	–	+ (128)	–
MC	–	+ (256)	–
BB	–	–	–
JV	–	+ (256)	–

* Antibody to EBNA in Burkitt lymphoma sera were performed through the courtesy of Dr. G. de Thè. EBNA antibody studies for the other sera were performed on WiL_2 cells as were all studies for antibody to RANA. Numbers in parenthesis represent the reciprocal of the highest dilution of sera positive for staining or precipitation.

(15). In addition, the patterns of nuclear staining were completely different in that EBNA staining was dense and clumpy involving the entire nucleus, whereas, RA nuclear antigen staining consisted of discrete, individual speckles as illustrated in Fig. 1.

Further studies which differentiated the RANA from EBNA are presented in Table II. The three prototype RA sera used in these studies were all positive for antibody to RA nuclear antigen by immunofluorescence and immunodiffusion but only two of the three had antibody to EBNA. The lack of relationship between the two nuclear antigens was also demonstrated by sera of patients with Burkitt's lymphoma and certain normal sera. In many instances, high titers of antibodies to EBNA were not associated with any detectable antibodies to RANA, either by immunofluorescence or immunodiffusion.

Discussion

In an earlier study (14), it was demonstrated that the soluble nuclear antigen reactive with serum antibody in RA sera was present in human lymphocyte cell lines WiL_2 and Raji but not present in a variety of normal human and animal tissues. It was postulated that the nuclear antigen might belong to a class of nuclear "derepressor" proteins present in rapidly proliferating cells. Another possibility was that the nuclear antigen might be a viral-induced protein since WiL_2 and Raji cell lines are B lymphocytes known to carry EBV. In other studies not reported here, we have determined that RA-reactive nuclear antigen is not present in other rapidly proliferating tissue culture cell lines, including monkey kidney cells (CV-1), human fibroblast cells (WI38), and human T lymphocytes (1301 and Molt4). The present studies suggest that it might be an EBV-associated antigen. This is supported by the demonstration that the nuclear antigen began to appear in human lymphocytes in the early stages of transformation induced by EBV and increased in intensity and in percentage of positive cells as transformation progressed. The nuclear antigen was present in human and simian lymphocytes transformed with EBV, but not in lymphocytes transformed with herpesviruses of simian origin. Human lymphocytes were not strictly necessary for the expression of this nuclear antigen, since it could be detected in simian lymphocytes transformed with EBV.

Our studies suggest that the RANA is different from EBNA. The antigens showed different patterns of nuclear staining and different reactivity to anti-complement and anti-IgG fluorescein reagents. Heat fixation was the best method of preserving the RA-associated antigen for its demonstration by immunofluorescence, a situation different from EBNA which is best demonstrated by fixation in cold acetone. Furthermore, certain sera contained antibodies to EBNA, but no antibodies to RA-associated antigen. RA antibodies are also precipitating antibodies whereas antibody to EBNA has not been demonstrated by precipitation reactions. Once induced, the RANA can be demonstrated to be present at all times in EBV-transformed cells and is therefore different from the EBV-induced transient nuclear antigen recently described (21).

These studies do not tell us if there is an etiological relationship between EBV infection and RA. If a relationship is present, specific host and other factors must play important roles since EBV has been implicated in such diverse diseases as Burkitt's lymphoma, nasopharyngeal carcinoma, and infectious mononucleosis. However, if a persistent infectious agent should be one of the factors causing RA, the role of EBV might merit further study.

Summary

Sera from approximately two-thirds of patients with rheumatoid arthritis contain an antibody which is reactive with a nuclear antigen present in human B-lymphocyte tissue culture cells. The immunological reaction can be demonstrated by precipitation and immunofluorescence. Evidence is presented that the reactive nuclear antigen is associated with Epstein-Barr (EB) virus-transformed lymphocytes. Normal human peripheral blood lymphocytes did not contain the nuclear antigen reactive with rheumatoid arthritis sera, but after infection with EB virus, they showed increasing amounts of reactive nuclear antigen as the cells were transformed into continuous lines. Several established human and simian lymphocyte cell lines known to carry EB viral genomes were shown to contain rheumatoid arthritis-associated nuclear antigen. Evidence is presented which suggests that the rheumatoid arthritis-associated nuclear antigen is different from the previously described EB nuclear antigen.

The authors with to acknowledge with gratitude the advice of Dr. Henry G. Kunkel during the course of this work and during the preparation of this manuscript.

Received for publication 12 December 1977.

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A NEW LYMPHOCYTE-ACTIVATING DETERMINANT LOCUS EXPRESSED ON T CELLS, AND MAPPING IN *I-C* SUBREGION*

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The *H-2* gene complex of the mouse is comprised of four distinct regions, *K* and *D*, marked by genes which control histocompatibility antigens, *S*, defined by a gene(s) for the Ss serum protein (C′4 component) and Slp allotype traits, and *I*, which include genes controlling immune responses, lymphocyte-activating determinants (*Lad*),[1] graft-versus-host reaction, immune response-associated (Ia) antigen, and a histocompatibility locus (1-3). The *I* region has been further divided into subregions, *I-A*, *I-B*, *I-J*, *I-E*, and *I-C*, which control different sets of specific immune responses or suppression (4).

I-C subregion was defined by specificity Ia.6 which differentiated $H\text{-}2^k$ (Ia.6−) and $H\text{-}2^d$ (Ia.6+) haplotypes for this subregion (2). *I-E* subregion was defined by specificity Ia.22, positive in $H\text{-}2^k$ and negative in $H\text{-}2^d$ (4). Recently, we identified a new private Ia specificity (Ia.23) of $H\text{-}2^d$ origin, also mapping in *I-E* subregion.[2] Sequential precipitation studies suggest that specificities Ia.7, 22 ($H\text{-}2^k$) and Ia.7, 23 ($H\text{-}2^d$) are expressed on products coded by *I-E* subregion gene(s).[3] That leaves Ia.6 as the only specificity mapping in the *I-C* subregion.

Previous investigations have shown that genes controlling products which cause stimulation in the mixed lymphocyte reaction (MLR) map in a number of different regions of the *H-2* gene complex (5, 6). The strongest MLR stimulation has been seen with *I* region differences. Specific loci controlling *Lad* were identified in the *I-A* and *I-C* subregions (7). Recently, we identified a *Lad* locus expressed exclusively on T cells and mapping in the *I-J* subregion (8). Previous MLR studies with the *I-C* subregion also included incompatibility at *I-J* and *I-E* subregions (9).

By using recombinant strains of mice which differ for *I-C* (including *S* and *G*) subregion (A and A.AL; B10.AM and B10.A(2R)) or combinations with only *I-C* incompatibility [(A × C3H.OL)F_1 and A.AL], we have attempted to test for the existence of a *Lad* locus in this subregion. Our results show that *I-C* subregion gene(s) code for MLR determinants on T cells. *I-C* subregion also code for genes involved in the alloantigen-mediated MLR suppressor factors (10, 11), α-gene

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* Supported by grants from the National Institute of Allergy and Infectious Diseases (AI 12734, AI 14764).

[1] *Abbreviations used in this paper:* AS, agarose and spleen cell absorbed, EDTA-treated; EHAA, modified Eagles-Hanks medium; FcR⁺, Fc receptor positive, Glφ, random linear terpolymer of L-glutamic acid, L-lysine, and L-phenylalanine; HBSS, Hanks balanced salt solution; Ia, immune response-associated; *Lad*, lymphocyte-activating determinant; MLR, mixed lymphocyte reaction; NMS, normal mouse serum.

[2] C. S. David et al. Manuscript submitted for publication.

[3] Cullen et al. Manuscript in preparation.

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for response to random linear terpolymer of L-glutamic acid, L-lysine, and L-phenylalanine (GLϕ) (12), determinants on Fc receptor positive (FcR$^+$)T cells (13) and possibly other T-cell functions.

Materials and Methods

Mice and Alloantisera. Inbred strains, recombinants, and F_1 hybrids used in this study were all produced in the Department of Genetics mouse colony at Washington University School of Medicine, St. Louis, Mo. A battery of anti-*Ia* and anti-*H-2* antisera were produced in our laboratories, as previously described (14).

Cell Preparation. Thymus cells, spleen cells, and lymph node cells were used as T-cell sources. Purification of T cells was performed by passing the lymph node cell suspensions through nylon wool columns (15).

Separation of B cells from spleen cells was performed as follows: spleen cells (5×10^7) were reacted with anti-Thy-1.2 serum (provided by the Transplantation Branch, National Institutes of Allergy and Infectious Diseases, Bethesda, Md.) at a final concentration of 10% antiserum in 5% fetal calf serum-containing RPMI 1640 medium. After 30 min at 37°C, the cells were washed twice with Hanks' balanced salt solution (HBSS). Rabbit complement was added and the mixture was incubated for an additional 40 min at 37°C, in a humidified atmosphere containing 5% carbon dioxide. For absorption of anti-Ia sera, bone marrow cells were used as a source of B cells (16).

Macrophages were collected by washing the normal mouse peritoneal cells with HBSS. Thereafter, the peritoneal cell suspension was placed into tissue culture dishes (Corning Glass Works, Corning, N.Y.) for 60 min and nonadherent cells removed. The adherent cells were collected and after one more 60 min incubation, were used as the source of macrophages.

Cytotoxic Test. Two-stage dye-exclusion microcytotoxic tests were performed as previously described (14).

Absorption of Anti-Ia Antisera. 100 λ of antiserum (B10.K × A.TL)F_1 anti-A (anti-I-C^d, S^d, G^d) was diluted 1/10 with a modified Eagles'-Hanks' medium (EHAA) (17) containing 2% human plasma, and adsorbed with 1×10^8 cells of (*a*) macrophages, (*b*) B cells (bone marrow cells), and (*c*) T cells, respectively, of B10.A(2R) (I-C^d) mice, at 37°C for 60 min. After incubation, supernates were assayed for their ability to inhibit MLR response by using B10.AM (I-C^k) T cells as responder and B10.A(2R) (I-C^d) T cells as stimulator.

Mixed Lymphocyte Reaction Assay. The medium employed in this assay was an EHAA medium supplemented with 2% human plasma. 5×10^5 viable responder cells were cultured with 1.0×10^6 mitomycin C-treated stimulator cells (18) in 0.2 ml of medium in microculture plates (Falcon 3040, BioQuest, BBL & Falcon Products, Becton, Dickinson & Co., Cockeysville, Md.). Incubation was performed at 37°C in a 5% carbon dioxide, 95% air, humidified atmosphere for 72 h. 2 μCi of [^{3}H]thymidine (sp. act 1.9 Ci per mmol, New England Nuclear, Boston, Mass.) was then added and the culture was harvested after a further 16 h. Within each experiment the mean and SD are calculated from three replicate wells. The experiments were repeated three to six times to check for repeatability of results and stimulation index.

Inhibition of MLR. MLR assays were done by the technique described above. 5×10^5 nylon wool-purified responder T cells were cocultured with 1.0×10^6 mitomycin C-treated, nylon wool-purified stimulator cells in the presence of 1.25% heat-inactivated alloantiserum, or normal control serum, in a total vol of 200 μl. Percent inhibition was calculated according to the following formula:

Percent inhibition =

$$\left(1 - \frac{\text{cpm of allogeneic culture in antiserum-cpm of syngeneic culture in NMS}}{\text{cpm of allogeneic culture in NMS-cpm of syngeneic culture in NMS}}\right) \times 100.$$

Treatment of Ia-Antisera and Complement. Nylon wool-purified T cells were reacted with antiserum or control normal mouse serum (NMS), at a final concentration of 10%, in RPMI-1640 containing 2.5% heat-inactivated human plasma. After incubation for 30 min at 37°C, the cells were washed and suspended in agarose- and spleen cell-absorbed, EDTA-treated (AS-absorbed) rabbit complement (19). After incubation for 90 min at 37°C, the dead cells were counted and separated from live cells on a Ficoll-Hypaque gradient. Dead cells in these tests were scored by a dye exclusion test. Only surviving cells, after the treatment by antisera and complement (and followed by mitomycin C treatment), were used as stimulator cells in these MLR tests. Culture media, cell numbers of stimulators and responders, the period of culture, and the method of harvesting were all the same as those described above. No antisera were added to the media for elimination studies. Percent inhibition was estimated by the formula:

Percent inhibition =

$$\left(1-\frac{\text{cpm of allogeneic culture of treated cells-cpm of syngeneic culture}}{\text{cpm of allogeneic culture-cpm of syngeneic culture}}\right)\times 100.$$

Results

Mixed Lymphocyte Reaction Across I-C Subregion. Strain combinations A-A.AL and B10.AM-B10.A(2R) were used in one-way MLR tests to detect *Lad* determinants controlled by the *I-C* subregion. These strains differ in *I-C*, *S*, and *G* regions (Table I). As shown in Table II, when lymph node cells were used as stimulators and responders, a significant MLR response was seen. In general, $I\text{-}C^d$ strains were better stimulators than the $I\text{-}C^k$ strains. To rule out the contribution of *S* and *G* regions (as well as *TL* and *Q* regions), (A × C3H.OL)F_1 animals were used as the responding strains and A.AL as the stimulating strains. Substantial stimulation was detected which should be due only to MLR determinants mapping in the *I-C* subregion. This experiment was repeated six times and similar results were obtained.

The Origin of the Stimulator Cell. Mitomycin C-treated stimulator cells from strain A and untreated responder lymph node cells from A.AL mice were cocultured to detect the population of cells stimulating across the *I-C* subregion. The stimulator cell populations used, were spleens, lymph nodes, thymuses, purified T cells and B cells. As shown in Table III thymus cells showed very weak stimulation and spleen cells displayed intermediate stimulation. Cocultures with the lymph node cells yielded stimulation index of 2.79.

Purified T cells and B cells were used in subsequent experiments. T cells were purified by nylon wool passage while B cells were purified by treatment with anti-Thy-1.2 serum. The stimulation index was about 1.05 when B cells were used. When nylon wool-purified T cells were employed as stimulators, the stimulation index increased to about 3.3. This result suggests that *Lad* determinants of *I-C* subregion are primarily expressed on T cells. This experiment was repeated three times with similar results.

Inhibition of MLR Response by Anti-Ia Sera. Nylon wool-purified T cells of (A × C3H.OL)F_1 hybrids were cocultured with nylon wool-purified mitomycin C-treated A.AL T cells in the presence of various anti-Ia sera (Table IV). Strong inhibition was observed in the presence of antisera A anti-A.AL (anti-$I\text{-}C^k$, S^k, G^k) and B10.S(9R) anti-B10.HTT (anti-$I\text{-}J^s$, $I\text{-}C^k$, S^k, G^k). Both of these antisera lack cytotoxic activity against unfractionated spleen cells but could contain antibodies against $I\text{-}C^k$ region products. However, antisera (C3H.Q × B10.D2)F_1 anti-AQR (anti-$I\text{-}A^k$, $I\text{-}B^k$, $I\text{-}J^k$, $I\text{-}E^k$) and C3H.OH anti-C3H.OL (anti-S^k, G^k), which lacked antibodies against antigens coded by *I-C* subregion, failed to give inhibition. These antisera could possibly contain antibodies to antigens coded

TABLE I
H-2 Gene Complex of Selected Recombinants

Strain	H-2 Haplotype	H-2 Regions									
		K	*A*	*B*	*J*	*E*	*C*	*S*	*G*	*D*	*TL*
A	*a*	*k*	*k*	*k*	*k*	*k*	*d*	*d*	*d*	*d*	*a*
A.AL	*al*	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*d*	*c*
C3H.OL	*ol*	*d*	*d*	*d*	*d*	*d*	*d*	*k*	*k*	*k*	*b*
C3H.OH	*o2*	*d*	*d*	*d*	*d*	*d*	*d*	*d*	*d*	*k*	*b*
B10.AM	*h3*	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*b*	*b*
B10.A(2R)	*h2*	*k*	*k*	*k*	*k*	*k*	*d*	*d*	?	*b*	*b*
B10.HTT	*t3*	*s*	*s*	*s*	*s*	*k*	*k*	*k*	*k*	*d*	*c*
B10.S(9R)	*t4*	*s*	*s*	?	*k*	*k*	*d*	*d*	*d*	*d*	*a*

TABLE II
MLR Responses of Lymph Node Cells among I-C Subregion Differing Strains

Responder	Stimulator*	Incompatible region	cpm of [^{3}H]TdR‡ (mean ± SD)	Stimulation indices
B10.AM	B10.AM	–	970.8 ± 131	–
B10.AM	B10.A(2R)	C, S, G	4,832.0 ± 420	5.0
B10.A(2R)	B10.A(2R)	–	3,201.6 ± 481	–
B10.A(2R)	B10.AM	C, S, G	5,442.9 ± 1,966	1.7
A	A	–	1,609.5 ± 224	–
A	A.AL	C, S, G	5,020.1 ± 439	3.1
A.AL	A.AL	–	2,381.6 ± 293	–
A.AL	A	C, S, G	7,918.5 ± 386	3.3
(A × C3H.OL)F_1	(A × C3H.OL)F_1	–	2,526.7 ± 174	–
(A × C3H.OL)F_1	A.AL	C	10,733.8 ± 709	4.2

* Mitomycin C-treated cells.

‡ Represents mean and SD of three replicate wells.

by *TL* and *Q* loci, but the stimulator and responder are compatible for these two regions. Further, anti-Thy-1.2 antiserum as well as anti-$H\text{-}2K^k$ and anti-$H\text{-}2D^d$ failed to inhibit. These results also suggest that MLR observed in the combination (A × C3H.OL)F_1 versus A.AL is also due to *I-C* gene products and that the antisera made across *I-C* incompatibility contain antibodies against *Lad* gene products of this region.

To confirm the above results with anti-$I\text{-}C^d$ sera, B10.AM cells were used as responders and B10.A(2R) cells as stimulators. In this combination *S* and *G* regions (and *TL* and *Q*) are also incompatible. All the antisera that could contain antibodies against $I\text{-}C^d$ region products inhibited the MLR. To test the target cell involved in this inhibition, antiserum (B10.K × A.TL)F_1 anti-A (anti-$I\text{-}C^d$, S^d, G^d) was absorbed with purified B cells, T cells, and macrophages, respectively. Antisera absorbed with bone marrow cells (B cells) and peritoneal macrophages of B10.A(2R) ($I\text{-}C^d$) still gave substantial levels of MLR inhibition. When the antiserum was absorbed with nylon wool-purified T cells from B10.A(2R) lymph nodes, no inhibition was observed. These results also suggest

TABLE III

MLR Responses of Lymph Node Cells Stimulated by Thymocytes, Spleen Cells, Lymph Node Cells, B Cells, and T Cells Incompatible for I-C Subregion

Responder (lymph node cells)	Stimulator*	cpm of [^{3}H]TdR Incorporation	
		Experiment‡ (mean ± SD)	Stimulation indices
A.AL	A.AL (Spleen)	4,571.6 ± 283	–
A.AL	A (Spleen)	10,015.1 ± 734	2.19
A.AL	A.AL (Thymus)	1,786.3 ± 481	–
A.AL	A (Thymus)	1,933.2 ± 260	1.08
A.AL	A.AL (Lymph node)	4,467.9 ± 235	–
A.AL	A (Lymph node)	12,480.6 ± 791	2.79
A.AL	A.AL (B Cells)§	4,950.1 ± 312	–
A.AL	A (B Cells)	5,218.3 ± 1,071	1.05
A.AL	A.AL (T Cells)‖	4,162.6 ± 223	–
A.AL	A (T Cells)	13,766.0 ± 1,942	3.30

* Mitomycin C-treated cells.

‡ Mean and SD of three replicate wells.

§ Prepared by treatment of spleen cells with anti-Thy-1.2 + C'. About 90% cells were immunoglobulin-positive cells.

‖ Prepared by nylon wool column. About 90% cells were Thy-1.2 antigen positive.

that *I-C* gene products are expressed on T cells and not on B cells and macrophages.

In an attempt to rule out the possibility that antibodies against *S* and *G* region products are involved in the above MLR inhibitions, the antisera were absorbed with C3H.OL lymphocytes. C3H.OL ($I\text{-}C^{d}$, S^{k}, G^{k}) removes the inhibiting capacity of (B10.K × A.TL)F$_1$ anti-A (anti-$I\text{-}C^{d}$, S^{d}, G^{d}) but not B10.S(7R) anti-B10.HTT (anti-$I\text{-}E^{k}$, $I\text{-}C^{k}$, S^{k}, G^{k}). Thus, removal of antibodies against the *S* and *G* regions does not affect inhibition of *I-C* incompatible MLR. These results also indicate that putative antibodies against *TL* and *Q* region antigens do not cause inhibition.

Elimination of Stimulating Cells with Anti-Ia +C'. We next investigated the ability of various anti-Ia sera to eliminate the stimulating cell population in the presence of complement. Nylon wool-purified T cells of B10.AM mice were incubated with anti-Ia sera and rabbit complement. The dead cells were eliminated by centrifugation on Ficoll-Hypaque and the viable T cells were used as stimulators. As shown in Table V antisera directed against $I\text{-}C^{k}$ region products eliminated the stimulating cells. Antiserum (B10.A(4R) × C3H.OL)F$_1$ anti-B10.K could not contain antibodies against *TL* or *Q* region antigens. Antisera directed against $I\text{-}A^{k}$, $I\text{-}B^{k}$, $I\text{-}J^{k}$, $I\text{-}E^{k}$, S^{k}, G^{k} had no effect. These results suggest that the *I-C* gene products are expressed on a subpopulation of T cells which does not express the other *I* subregion products. As expected anti-$H\text{-}2K^{k}$, anti-$H\text{-}2D^{b}$, and anti-Thy-1.2 eliminated the MLR response.

TABLE IV

Effects of Anti-Ia Sera on MLR Responses between A.AL T Cells as Stimulator and T Cells of (C3H.OL × A)F$_1$ Hybrids as Responder

Responder*	Stimulator‡	Treatment§	Region	[^{3}H]TdR Incorporation (mean ± SD)‖	Inhibition¶
					%
A × C3H.OL	A × C3H.OL	NMS		635.0 ± 120	–
A × C3H.OL	A.AL	NMS		3,281.3 ± 278	–
A × C3H.OL	A.AL	B10.S(7R) Anti-B10.HTT	(E, C, S, G)^k	1,660.5 ± 234	61
A × C3H.OL	A.AL	A Anti-A.AL	(C, S, G)^k	2,071.1 ± 563	46
A × C3H.OL	A.AL	B10.S(9R) Anti-B10.HTT	J^s (C, S, G)^k	1,254.7 ± 310	77
A × C3H.OL	A.AL	(C3H.Q × B10.D2)F$_1$ Anti-AQR	(A, B, J, E)^k	2,890.9 ± 237	15
A × C3H.OL	A.AL	A.TL Anti-A.AL	K^k	2,658.3 ± 325	24
A × C3H.OL	A.AL	(B10.K × A.TL)F$_1$ Anti-A	(C, S, G)^d	2,850.3 ± 180	26
A × C3H.OL	A.AL	C3H.OH Anti-C3H.OL	(S, G)^k	2,973.1 ± 268	12
A × C3H.OL	A.AL	(B10.AKM × ASW)F$_1$ Anti-A.TH	D^d	3,146.0 ± 519	5
A × C3H.OL	A.AL	Anti-Thy.1.2		3,011.7 ± 271	10

* Nylon wool-purified T cells.

‡ Mitomycin C-treated T cells.

§ Serum at 1.25% final dilution in the culture.

‖ Mean and SD from three replicate wells.

¶ Inhibition $= \left(1 - \frac{\text{allogeneic MLR in anti-Ia sera-syngeneic MLR in NMS}}{\text{allogeneic MLR in NMS} - \text{syngeneic MLR in NMS}}\right) \times 100.$

Discussion

Specificity Ia.6 was defined as a private specificity of *H-2^d* haplotype mapping in the *I-C^d* subregion. Haplotype *H-2^a* was positive for Ia.6 which suggested the expression of *I-C^d* segment in that strain. Ia.6 specificity exhibited several unique qualities. Only 15–20% of lymph node cells were lysed by anti-Ia.6 and the antibodies were detected only in the early bleedings and were IgM in nature (20). The antisera reacted with some T-cell lymphomas. After the original antisera was exhausted we were unable to produce other sera with cytotoxic activity. Further, we were unable to detect Ia.6 by immunoprecipitation assay by using spleen cells (Cullen, unpublished observations). These results suggested that Ia.6 may be a T-cell specificity. Recent results indicate that Ia.6 is expressed on a subpopulation of T cells from *I^d* strains mapping in *I-C*. *H-2^a* haplotypes and the recombinants derived from it express this antigen.

Recent results on the mapping of Ia.22 and Ia.23 further clarify the definition

TABLE V

Anti-Ia Elimination of MLR Response Among B10.A(2R) T Cells as Responder and B10.AM as Stimulator

Responder*	Stimulator‡	Pretreatment§	Region	[^{3}H]TdR Incorporation (mean ± SD)	Inhibition
					%
B10.A(2R)	B10.A(2R)	NMS + C′	(−)	4,324.3 ± 459	−
B10.A(2R)	B10.AM	NMS + C′	(−)	7,386.0 ± 521	−
B10.A(2R)	B10.AM	(B10.A(4R) × C3H.OL)F$_1$ Anti-B10.K + C′	$(B, J, E, C)^k$	4,056.6 ± 108	100
B10.A(2R)	B10.AM	B10.S(7R) Anti-B10.HTT + C′	$J^s(C, S, G)^k$	4,418.5 ± 317	97
B10.A(2R)	B10.AM	C3H.OH Anti-C3H.OL + C′	$(S, G)^k$	7,035.8 ± 561	11
B10.A(2R)	B10.AM	(A.TH × B10.HTT)F$_1$ Anti-A.TL + C′	$(A, B, J)^k$	6,703.2 ± 837	22
B10.A(2R)	B10.AM	B10.A(3R) Anti-B10.A(5R) + C′	J^k	6,814.1 ± 710	19
B10.A(2R)	B10.AM	A.TL Anti-A.AL + C′	K^k	3,976	100
B10.A(2R)	B10.AM	Anti-Thy-1.2 + C′		3,033	100

* Nylon wool-purified T cells.

‡ Mitomycin C-treated T cells.

§ Stimulator cell treated with $^1/_{10}$ serum plus AS-absorbed rabbit complement, then washed.

of *I-E* and *I-C* subregions. Sequential immunoprecipitation experiments suggest that Ia.22 and Ia.7 are expressed on the same molecule.[3] Further, Ia.23 and Ia.7 are also expressed on the same molecule.[2] The map positions of specificities Ia.7, 22 and 23 seem to fall within the *I-E* subregion, Ia.7 being a public specificity while Ia.22 and 23 are private specificities of I^k and I^d strains, respectively. Thus specificities with predominant expression on B cells map within the *I-E* subregion while Ia.6 expressed on T cells map within the *I-C* subregion.

The mixed lymphocyte reaction studies in strain combination of A-A.AL and B10.AM-B10.A(2R) indicate a *Lad* locus controlled by the *I-C* subregion. When (A × C3H.OL)F$_1$ is used as responder and A.AL as stimulator, the incompatibility should only be at *I-C* subregion. Antisera made across *I-C* incompatible strains which do not exhibit cytotoxic activity on spleen cells can inhibit the MLR reaction. Even though some of these antisera could contain antibodies against gene products of *TL* and *Q* regions, they do not seem to be involved in the inhibition. The antibodies are directed against the MLR determinants on the stimulator cells. Further, absorption of these antisera with purified T cells removes the inhibiting ability while purified B cells and macrophages do not seem to play a role.

Our results and those of others suggest that *I-C* subregion gene(s) codes for products expressed selectively on a subpopulation of T cells. The following immunological traits have been mapped to the *I-C* subregion; (*a*) α-gene for response to GLϕ; (*b*) gene controlling generation and acceptance of alloantigen-mediated suppressor factors; (*c*) determinants on FcR^+ T cells; (*d*) specificity Ia.6, and (*e*) MLR simulating determinants. The number of genes involved in the control or regulation of the above phenomena remain to be seen.

Summary

A new lymphocyte-activating determinant (*Lad*) locus expressed on T cells was identified, mapping in the *I-C* subregions of $H\text{-}2^k$ and $H\text{-}2^d$ haplotypes. The mixed lymphocyte reaction stimulation could be inhibited by anti-Ia sera made in strains incompatible for this chromosomal segment. Experiments with purified lymphocyte cell populations suggested that this *Lad* locus was expressed on T cells. Further, only purified T cells were able to remove the inhibiting activity from the anti-Ia sera. *I-C* subregion gene(s) seem to code for products selectively expressed on a subpopulation of T cells.

The authors are indebted to Dr. Donald Shreffler for his advice and critical review of the manuscript. We also thank our other colleagues who read the manuscript and gave us suggestions. The expert technical assistance of Brian Neely is acknowledged.

Received for publication 6 January 1978.

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SUPPRESSION OF THE MIXED LYMPHOCYTE REACTION IN MAN BY A SOLUBLE T-CELL FACTOR

Specificity of the Factor for both Responder and Stimulator*

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We have previously reported the occurrence of suppressor T cells of the mixed lymphocyte reaction (MLR)[1] in a human leukocyte antigen (HLA) Dw2 homozygous woman, J.H., who failed to respond to the allogeneic cells of her husband, W.H. (1, 2). When J.H. suppressor T cells were cocultured in MLRs between responder cells and unrelated, irradiated stimulator cells, they only inhibited the responses of individuals who shared the HLA-Dw2 specificity with J.H. Moreover, only when W.H. or a few other stimulator cell types were present was J.H. suppression of MLR responses detected. Thus, the J.H. suppressor T cell appeared to be specific for determinants in the irradiated stimulator cells as well as *D* locus products in the responder.

We now report that a soluble factor or factors, released into the supernate of the MLR by J.H. T cells, also mediates this suppression. Like the cell from which it is derived, the factor is highly specific for HLA-D products in the responder cell and partially specific for the W.H. stimulator cell.

Materials and Methods

Blood samples were obtained from a panel of healthy persons known to be homozygous or heterozygous for specific HLA antigens. All experiments were performed with lymphocytes isolated from fresh venous blood.

Preparation of MLR Supernates. As described previously (1, 2), mononuclear leukocytes from defibrinated blood were collected by Ficoll-Hypaque gradient centrifugation, washed, and then resuspended at 1 × 10^6 cells/ml in RPMI-1640 medium (Grand Island Biological Co., Grand Island, N.Y.) containing 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 10% pooled type A human serum. Stimulating cells were irradiated in a ^{137}cesium irradiator (Mark I model 24 irradiator; J. L. Shepherd, & Associates, Glendale, Calif.) with a dose of 6,000 rads to abolish their capacity to proliferate and to make the reaction unidirectional.

For preparation of suppressor factor, mixed lymphocyte cultures were carried out in loosely capped 15 ml round bottomed plastic test tubes (BioQuest, BBL, & Falcon Products, Becton, Dickinson & Co., Cockeysville, Md.) with 3 × 10^6 J.H. cells and 3 × 10^6 irradiated W.H. cells in a vol of 6 ml. Up to 10 replicate cultures were carried out simultaneously in air/5% CO_2 at 37°C.

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* Supported by a grant from the Kroc Foundation (Santa Ynez, Calif.) and National Institutes of Health grant AI 11313.

‡ Supported by an American Cancer Society, California Division postdoctoral fellowship.

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[1] *Abbreviations used in this paper*: HLA, human leukocyte antigen; MLR, mixed lymphocyte reaction; PBS, phosphate-buffered saline.

Control cultures consisting of J.H. cells alone (0.5 × 10^6/ml) or J.H. and an unrelated irradiated cell (C.O.) were carried out simultaneously. After 48 h, the culture tubes were centrifuged at 400 *g* for 15 min at room temperature, after which the supernatant fluids were carefully withdrawn and pooled in 50 ml conical polypropylene tubes (Corning Glass Works, Science Products Div., Corning, N.Y.). To assure that no cells remained, pooled supernates were recentrifuged at 400 *g* for 15 min.

Assessing the Effects of Supernates on the MLR. To determine if the pooled supernates had any effect on the MLR, Ficoll-Hypaque enriched mononuclear leukocytes were suspended at 5 × 10^6 per ml in RPMI-1640 medium supplemented as indicated above. 50,000 responder cells (0.01 ml) were cultured with 50,000 irradiated stimulator cells (0.01 ml) in round bottom microtiter trays (Linbro Chemical Co., New Haven, Conn.) with either 0.15 ml supplemented medium or 0.15 ml supernate. Cultures, prepared in triplicate or sextuplicate, were incubated in air/5% CO_2 for 6 days at 37°C. [^{3}H]thymidine (New England Nuclear Corp., Boston, Mass.) was then added, 1 μCi per well, and the plates harvested in a Multiple Sample Harvester (MASH II, Microbiological Associates, Walkersville, Md.) 18 h later.

Separation of T Cells and B Cells. Peripheral blood mononuclear leukocytes were enriched for either T lymphocytes or B lymphocytes as previously described (1) on the basis of differential affinity for anti-immunoglobulin coated on plastic flasks. In this method 2 ml of Cohn Fraction II immunoglobulin (2 mg/ml) and soluble dicarbodiimide (1 mg/ml) in phosphate-buffered saline (PBS) were incubated in a 25 cm^2 polystyrene tissue culture flask (Corning Glass Works) for 1 h at room temperature. After the flask was washed with PBS-5% fetal calf serum, 2 ml of a 1:20 dilution of rabbit anti-human immunoglobulin was added and incubated for 30 min at room temperature before washing. Under these conditions monocytes as well as B cells adhered to the flask. The non-adherent T cells were decanted and the B cells removed after 2 h incubation at 37°C with RPMI-50% human A serum containing 1.25 mM EDTA. The T cells were measured in the two fractions by rosetting with sheep erythrocytes (1). The B cells were measured by staining with fluorescein-conjugated anti-Ig as described previously (3). Cell recovery was 75–90%, with T-cell fractions consisting of 85–90% rosetting cells and B-cell fractions consisting of 80–90% Ig-positive cells.

Results

Evidence that a Soluble Suppressor Factor is Released into the MLR Supernate by J.H. and that it is Specific for HLA Dw2 in the Responder. J.H. possesses the HLA antigens A2,3/B7,7/Dw2,2/DRw2,2. She shares no HLA antigens with her husband, W.H., who is HLA A11,28/B35,35/Dw1,-/DRw1,4. As shown in Table I, in the presence of medium alone, all of the responders tested proliferate vigorously in response to the irradiated stimulator, W.H. In the presence of supernate from a 48-h culture between J.H. and W.H., however, the responses of some donors were markedly inhibited. Of those individuals whose responses to W.H. were suppressed, all but one is positive for HLA-Dw2. The single exception is the cell R.K., which although not typed as HLA-Dw2 does share with J.H. the Dw2 associated Ia-like specificity DRw2, according to the criteria of the Seventh International Histocompatibility Workshop (B. Colome and R. Payne, personal communication).

Possession by responder cells of HLA-B7, an antigen in strong linkage disequilibrium with HLA-Dw2, is neither necessary for suppression nor sufficient for suppression in the absence of Dw2. For example, J.R., who is positive for Dw2 but negative for B7, is suppressed, but H.K., who possesses B7 but lacks Dw2, is not suppressed.

Results similar to those shown in Table I were obtained in five consecutive experiments in which 3 × 10^6 J.H. cells were cultured with 3 × 10^6 irradiated W.H. cells in a vol of 6 ml for 48 h. That is, a soluble suppressor factor was

TABLE I

The J.H. Suppressor Factor: Requirement for HLA-Dw2 in the MLR Responder Cell

Responder					Response to W.H. in the presence of medium*	Response to W.H. in the presence of J.H./W.H. supernate*	
HLA	A	B	Dw	DRw			*%Δ cpm*
C.L.	2,3	7,7	2,2	2,2	43,847 ± 3,794	14,433 ± 1,451	−67
T.I.	1,3	7,7	2,2	2,2	37,610 ± 4,599	11,112 ± 908	−71
T.L.	3,3	7,7	2,2	2,2	81,883 ± 6,729	23,682 ± 2,399	−71
J.L.	1,2	7,8	2,3	2,3	38,895 ± 3,076	20,684 ± 1,201	−47
R.L.	1,2	7,8	2,3	2,3	20,818 ± 1,459	10,362 ± 1,368	−50
R.G.	1,2	7,8	2,3	2,3	33,836 ± 2,065	17,073 ± 3,821	−50
J.R.	1,10	8,18	2,3	2,3	66,953 ± 7,386	41,942 ± 4,722	−37
E.G.	3,30	7,15	2,4	2,4	31,004 ± 3,463	14,260 ± 2,847	−54
W.B.	2,29	7,12	2,7	2,7	24,610 ± 2,715	14,103 ± 916	−43
M.K.	1,2	21,35	1,-	1,-	23,903 ± 3,316	30,773 ± 2,148	+29
C.O.	1,2	13,40	1,-	1,5	48,945 ± 2,778	44,413 ± 2,060	−9
H.K.	1,2	8,7	3,-	3,7	74,875 ± 4,110	67,550 ± 4,552	−10
C.H.	1,2	8,40	4,6	4,6	37,025 ± 5,905	38,111 ± 4,827	+3
L.W.	1,11	8,35	3,-	ND‡	80,316 ± 7,687	79,514 ± 9,376	−1
S.F.	2,24	13,27	4,-	4,-	53,116 ± 6,255	58,976 ± 4,847	+11
D.B.	9,14	12,32	4,-	4,7	27,366 ± 1,621	44,150 ± 2,910	+61
B.C.	1,29	17,35	5,6	5,6	67,351 ± 7,549	73,590 ± 5,162	+9
D.S.	3,29	27,40	5,-	1,5	29,641 ± 2,417	42,624 ± 4,705	+44
S.P.	3,29	21,12	7,-	3,7	31,835 ± 2,131	29,538 ± 2,644	−8
R.K.	1,28	5.2,14	-,-	2,5	72,214 ± 5,350	54,440 ± 6,406	−25

* Responses in cpm represent the means of six experiments ± standard error.

‡ ND, not done.

released into the supernate of the MLR, and this factor only inhibited the responses of Dw2-positive individuals. In earlier experiments, however, nonspecific suppression was observed in 96- and 144-h cultures and in 48-h cultures in which the concentrations of J.H. and W.H. cells had been increased fourfold (data not shown). No suppression was observed when an 18 h culture between J.H. and W.H. was tested (data not shown). On the basis of these observations we concluded that 48-h cultures between J.H. at 0.5 × 10^6 cells/ml and W.H. at 0.5 × 10^6 cells/ml were optimal for generation of specific suppressor factor. These conditions were duplicated in all subsequent experiments.

Antigen Specificity of the J.H. Suppressor Factor. We next tested the effects of the 48-h culture J.H./W.H. on the responses of the same donor panel to stimulator cells other than W.H. 10 such stimulators, chosen from a panel of HLA A, B, D typed cells, were tested. The responses of non-Dw2 individuals to these cells were not suppressed (data not shown). The effects of the same supernate on the responses of three Dw2-positive responders to these stimulator cells are shown in Table II. It is apparent that T.L., a cell homozygous for Dw2, is maximally suppressed in its response to W.H., although her responses to several other cells are also inhibited. Two Dw2 heterozygous responder cells are also inhibited maximally when W.H. is the stimulator cell and only slightly when other stimulator cells are tested.

TABLE II

*Specificity of the J.H. Suppressor Factor for the Stimulating Cell in the MLR**

Stimulator					Effect of the J.H. suppressor factor on mixed lymphocyte responses by:		
HLA	A	B	Dw	DRw	TL (Dw2,2)	JL (Dw2,3)	EG (Dw2,4)
						%	
W.H.	11,28	35,35	1,-	1,4	-71	-47	-54
S.D.	1,31	17,12	7,-	ND‡	-57	-27	-7
S.N.	2,24	21,35	-,-	5,4 × 7	-46	-11	-32
B.C.	1,29	17,35	5,6	5,6	-42	+3	-6
S.F.	2,24	13,27	4,-	4,-	-58	-3	-10
E.G.	3,30	7,15	2,4	2,4	-33	+8	ND‡
L.W.	1,11	8,35	3,-	ND‡	-17	+14	+48
H.K.	1,2	8,7	3,-	3,7	+7	+34	+20
W.B.	3,29	7,12	2,7	2,7	+13	+18	+10
C.O.	1,2	13,40	1,-	1,5	-17	+35	+40

* Supernate from a 48-h culture between J.H. and irradiated W.H. cells was tested for the capacity to inhibit the responses of 3 Dw2-positive cells to a panel of 10 unrelated stimulator cells. Results are expressed as the % change (either inhibition [-] or enhancement [+]) in baseline unidirectional MLRs.

‡ ND, not done.

TABLE III

*Failure to Obtain J. H. Suppressor Factor in an MLR between J.H. and C.O.**

Responder		Response to C.O. in the presence of:			Response to W.H. in the presence of:		
HLA	Dw	Medium	J.H./C.O. Supernate		Medium	J.H./C.O. supernate	
				%Δ cpm			*%Δ cpm*
C.L.	2,2	89,515 ± 7,833	99,977 ± 7,116	+12	43,847 ± 3,304	62,838 ± 5,894	+43
T.I.	2,2	58,344 ± 4,907	76,963 ± 5,442	+32	37,610 ± 4,805	38,425 ± 3,516	+2
R.L.	2,3	68,344 ± 5,938	86,963 ± 6,778	+27	20,818 ± 2,691	24,273 ± 2,028	+17
E.G.	2,4	81,656 ± 6,372	115,075 ± 10,097	+41	31,004 ± 1,917	37,737 ± 4,646	+22
W.B.	2,7	55,725 ± 6,573	61,002 ± 4,796	+10	70,539 ± 6,251	82,331 ± 8,175	+17
M.K.	1,-	27,320 ± 3,745	39,416 ± 5,248	+44	23,903 ± 1,905	34,708 ± 2,698	+45
H.K.	3,-	72,466 ± 6,069	94,224 ± 5,154	+30	74,875 ± 7,814	76,167 ± 5,812	+2
S.F.	4,-	54,711 ± 4,620	70,403 ± 7,386	+29	53,116 ± 5,338	62,371 ± 8,016	+17
B.C.	5,6	41,569 ± 2,450	77,136 ± 4,792	+86	29,641 ± 2,790	40,943 ± 5,227	+38
S.P.	7,-	43,152 ± 5,512	66,473 ± 6,489	+54	67,351 ± 5,100	92,222 ± 7,526	+37

* Peripheral blood lymphocytes from J.H. were incubated for 48 h with irradiated CO cells (HLA A1,2/B13,40/Dw1,-/DRw1,5), and the supernate was tested for its capacity to suppress the MLR responses to C.O. and W.H. Results represent the mean of triplicate cultures ± standard error.

Effects of a Supernate Obtained from an MLR between J.H. and a Stimulator Cell other than W.H. It was of interest to determine whether or not generation of the J.H. suppressor factor required the presence of W.H. as the irradiated stimulator cell. Therefore, a 48-h supernate was prepared from an MLR between J.H. and an unrelated irradiated cell, C.O. (HLA A1,2/B13,40/Dw1,-/DRw1,5). As shown in Table III, this supernate not only failed to inhibit the responses to W.H. and C.O. but actually enhanced the responses of the entire panel to these stimulator cells. A 48-h supernate from J.H. cultured in the absence of other cells had no effect on the MLR responses of our panel (data not shown).

Evidence that the J.H. Suppressor Factor is Released by T Cells. 3×10^6

TABLE IV

*Evidence that the J.H. Suppressor Factor is a T-Cell Product**

Responder		%Δ Response to W.H. in the presence of:	
HLA	Dw	T-Cell supernate	B-Cell supernate
C.L.	2,2	−58	−14
T.I.	2,2	−41	−12
R.L.	2,3	−37	−8
E.G.	2,4	−39	−9
M.K.	1,-	+5	+13
H.K.	3,-	+2	+12
S.F.	4,-	+4	+14
B.C.	5,6	+8	+15

* Peripheral blood lymphocytes, enriched for either T cells or B cells, were incubated with irradiated W.H. cells for 48 h and the supernates tested for MLR suppressor factor. Results are expressed as % change (either inhibition [−] or enhancement [+]) in the base line unidirectional MLR.

J.H. T-enriched cells or B-enriched cells were cultured with 3 × 10^6 irradiated W.H. cells in a vol of 6 ml for 48 h, and the supernates tested for suppressive activity. As shown in Table IV, significant suppression of Dw2 responders was obtained with supernate from a T-cell enriched culture but not supernate from a B-cell enriched culture.

Discussion

These results demonstrate that a soluble factor or factors suppressive of the MLR is generated when J.H. lymphocytes are cultured with the irradiated cells of her husband, W.H. Experiments with lymphocyte fractions enriched for either T cells or B cells suggest that the source of the J.H. suppressor factor is a T cell. J.H. T cells have been shown previously to inhibit the MLR (1, 2), and it is possible that the same cells are the source of suppressor factor.

The remarkable features of the J.H. suppressor factor are its specificity both for the MLR stimulator cell and for the MLR responding cell. The stimulator specificity parallels that observed with J.H. cells in MLR suppression (1, 2). The factor is generated in the combination J.H./W.H., and it is not generated when J.H. is cultured alone or with another irradiated cell, C.O. It suppresses the response to W.H. and certain other stimulator cells though these do not share HLA private specificities. In some instances suppression of the response to a broader spectrum of stimulating cells was seen (Table II).

The factor also shows specificity for the HLA-D region of the responding cell. The specificity is for HLA-Dw2, which is the HLA D type of J.H. A cell which lacked Dw2 but was nonetheless suppressed was serologically typed for the Dw2 associated Ia-like specificity, DRw2. These results, therefore, confirm the previous observation that sharing of D region products is required between the J.H. suppressor T cell and the target responder cell (2). This is the first report of such genetic restriction in immune cell interaction in man and the first example of a human T-cell factor that mediates such an interaction.

There are, however, a number of examples in mice. Thus, T-cell factors have been described that mediate interactions between T cells and T cells (4, 5), T cells, and B cells (6–8), and T cells and macrophages (9). Some murine T-cell factors are antigen specific (4, 8, 9, 10–12), and some only affect responders which share immune response region identity with the factor donor (4, 13). Although their exact nature is uncertain, it has been suggested that those factors which show antigen specificity may be related to the T-cell antigen receptor (14).

The murine factor described by Rich and Rich is similar to the J.H. factor in that it mediates suppression of the MLR and is generated by alloantigen-activated T cells (15). It is highly specific for responder cells in that it only affects the responses of strains histocompatible for the immune response region of the H-2 complex (to the right of and including *I–C*) (13). The murine MLR suppressor factor differs from the J.H. factor in its apparent lack of specificity for antigen (stimulating cell), but the range of specificity tested in inbred mice may be much less than in these experiments with outbred humans.

Experiments are in progress to determine both the target of the J.H. suppressor factor and the immunochemical nature of the factor. Antigen-specific factors in the mouse have been shown to have Ia antigen specificities (4, 8, 16–18). The presence of such specificities in the J.H. factor would add to the accumulating evidence that the *HLA-D* region in man is analogous to the murine immune response region.

Summary

J.H., an HLA-Dw2 homozygous multiparous woman, fails to respond to her husband, W.H. (HLA Dw1,-) in the unidirectional mixed lymphocyte reaction. T cells from J.H. were previously shown to suppress the responses of Dw2-positive cells but not Dw2-negative cells to W.H. We now report that a soluble factor released into the supernate of the mixed lymphocyte reaction by J.H. T cells, mediates this suppression. Like the cell from which it is derived, the factor is highly specific for HLA Dw2 in the responder cell and partially specific for the stimulatory alloantigen.

We thank Doctors Rose Payne and Beth Colombe for HLA A, B, and DR typing of the donor panel; Marion Batey and Linda Siri for excellent technical assistance; and J.H. and W.H. for their continued cooperation.

Received for publication 3 January 1978.

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EFFECT OF PSEUDOTYPE ON ABELSON VIRUS AND KIRSTEN SARCOMA VIRUS-INDUCED LEUKEMIA*

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Several RNA tumor viruses cause leukemia after inoculation into mice. The Abelson leukemia virus (A-MuLV)[1] (1) causes a rapidly progressive lymphoid leukemia, originating in the bone marrow or lymph nodes. In addition, A-MuLV quantitatively induces a leukemic transformation of hematopoietic cells in vitro (2, 3). The leukemic cells isolated in vivo or in vitro have the properties of neither mature T nor B cells (4), and are perhaps best classified as null cells. The Kirsten sarcoma virus (KiSV) causes an erythroid leukemia (5).

A-MuLV and KiSV quantitatively transform NIH/3T3 cells allowing an accurate determination of virus titer. Both viruses are defective and require a competent helper virus to replicate. Viral transformed nonproducer NIH/3T3 cells free of helper virus have been isolated (5, 6). Replicating defective virus can be rescued from these cells after superinfection with a competent helper virus. The super-infecting helper virus determines the pseudotype (tropism and envelope glycoprotein antigens) of the defective virus. The replication of RNA tumor viruses is restricted by the *Fv-1* locus of the mouse. Two codominant genetic alleles, $Fv\text{-}1^n$ and $Fv\text{-}1^b$, determine susceptibility to infection by RNA tumor viruses. Fv-1^{nn} mice are susceptible to infection by N- or NB-tropic viruses but are resistant to B-tropic viruses, while Fv-1^{bb} mice are susceptible to B- or NB-tropic viruses but are resistant to N-tropic viruses (7, 8).

I have rescued A-MuLV and KiSV from nonproducer cells with several helper viruses to study the effect of virus pseudotype on leukemogenesis. I found that the helper virus profoundly affects the incidence of erythroid leukemia caused by KiSV and the lymphoid leukemia caused by A-MuLV. The helper virus also influences the efficiency of A-MuLV-induced in vitro transformation of hematopoietic cells. The effect of the helper virus is independent of the *Fv-1* locus of the mouse.

Materials and Methods

Viruses and Cells. Clone 1 (9) of NB-tropic Moloney leukemia virus (M-MuLV) was obtained from Dr. D. Baltimore, M.I.T., while NB-tropic Rauscher leukemia virus (R-MuLV) (10) was

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* Supported by grants CA-18662 and CA-06516 from the National Institutes of Health.

‡ Leukemia Society of America Scholar.

[1] *Abbreviations used in this paper:* A-MuLV, Abelson murine leukemia virus; F-MuLV, Friend helper murine leukemia virus; Fv-1, Friend virus 1 genetic locus; FFU, focus-forming units; FMR, Friend, Moloney, and Rauscher group of murine leukemia viruses; Ki-MuLV, Kirsten murine leukemia virus, KiSV; Kirsten sarcoma virus; M-MuLV, Moloney murine leukemia virus; moi, multiplicity of infection; N35-MuLV, clonal isolate number 35 of WN1802N murine leukemia virus; PFU, plaque-forming units; R-MuLV, Rauscher murine leukemia virus; SFFV, spleen focus-forming virus.

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obtained from Dr. G. Todaro, N.C.I. The R-MuLV induced erythroid leukemia in vivo. N-tropic Friend polycythemia virus (SFFV[F-MuLV]) (11) was a gift of Dr. R. Steeves, Albert Einstein College of Medicine. The helper virus (F-MuLV) (12) was freed from spleen focus-forming virus (SFFV) by end point dilution on NIH/3T3 cells. A tissue culture passage of N-tropic Kirsten leukemia virus (Ki-MuLV) (13) which did not induce erythroid leukemia in vivo, was obtained from Dr. J. Stephenson, N.C.I. A clonal isolate (N35-MuLV) of an N-tropic endogenous virus (WN1802N) (14) isolated from the spleen of a normal elderly BALB/c mouse was a gift of Dr. P. Jolicoeur, M.I.T.

To obtain stocks of KiSV or A-MuLV as pseudotypes with different helper viruses, a clonal isolate of KiSV-transformed NIH/3T3 cells (5), or a clonal isolate of A-MuLV-transformed NIH/3T3 cells (ANN-1) (6) was infected with various N- or NB-tropic helper leukemia viruses. About 10^6 KiSV- or A-MuLV-transformed nonproducer cells were infected with a competent helper virus in the presence of polybrene (4 μg/ml) (Aldrich Chemical Co., Inc., Milwaukee, Wis.). Media was collected 1 wk later and passed through a 0.45-μm filter (Nalge Co., Nalgene Labware Div., Rochester, N.Y.). The titer of the defective transforming virus, expressed as focus-forming units per ml (FFU/ml), was determined on NIH/3T3 cells (5, 6). The cells were then ultraviolet-irradiated and overlaid with XC cells to determine the titer of helper virus (15), expressed as plaque-forming units per ml (PFU/ml). The NIH/3T3 and XC cells were grown in Dulbecco's modified Eagle's medium containing 10% calf serum (Colorado Serum Co. Denver, Colo.) at 37°C in a humidified atmosphere containing 10% CO_2.

Mice. C57/L ($Fv\text{-}1^{nn}$) and DBA/2De ($Fv\text{-}1^{nn}$) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine), while Swiss mice (CD-1) ($Fv\text{-}1^{nn}$) were purchased from Charles River Breeding Laboratories (Wilmington, Mass.). The Swiss mice were bred randomly, while the inbred strains were maintained by brother-sister crosses.

Induction of Erythroid Leukemia. Newborn Swiss mice were inoculated intraperitoneally with 0.1 ml of filtered virus stock. 3-4 wk later, the animals were killed and autopsied. Spleens were sectioned sagitally and touch preparations were made and treated with Wright-Giemsa stain. Animals with erythroblastic leukemia had grossly enlarged spleens which often ruptured, causing the death of the animal. More than 30% of the cells in areas of erythroid leukemia were proerythroblasts or basophilic erythroblasts, and occasional trinucleate forms were seen (5). The leukemia could be induced in hypertransfused mice.

To assay for the replication of KiSV, spleens were shredded through a wire grid and a 10% (wt/vol) cell suspension was made in medium. After removing the cells by low-speed centrifugation, the supernate was passed through a 0.45-μm membrane filter and assayed on NIH/3T3 cells (5).

Induction of Abelson Leukemia. Newborn Swiss or DBA/2 mice were inoculated with 0.1 ml of filtered virus stock. The animals were examined daily for the presence of enlarged lymph nodes, cranial tumors, or paralysis. Mice with these findings were killed and autopsied. Animals scored as having Abelson leukemia had gross and histologic evidence of lymphoblastic infiltration of the marrow, lymph nodes, and/or meninges with no involvement of the thymus (1).

Abelson Virus-Induced Hematopoietic Cell Transformation. The femoral bone marrow cells from 1 to 2-mo-old C57/L mice were collected in RPMI 1640 medium (Grand Island Biological Co., Grand Island, N.Y.) containing 20% heat (56°C) inactivated fetal calf serum, and 5×10^{-5} M 2-mercaptoethanol. The nucleated cells were diluted to 1×10^6 cells/ml and polybrene was added to a concentration of 2 μg/ml. The filtered virus stock (0.5 ml) was added to 1.5 ml of cell suspension and incubated at 37°C for 3 h. The cells were collected by low speed centrifugation (175 *g*) for 15 min and resuspended in RPMI 1640 medium containing 20% heat inactivated fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 0.3% (wt/vol) agarose (Type II; Sigma Chemical Co., St. Louis, Mo.) at a concentration of 1×10^6 nucleated cells/ml. The cell suspensions (1.5 ml) were added to 30-mm plastic dishes (Nunc Products, Vanguard International, Red Bank, N.J.) and incubated at 37°C in a humidified atmosphere containing 8% CO_2. The cultures were fed with 1 ml of agarose medium at 5 and 10 days postinfection. Lymphoid colonies containing more than 1,000 cells were quantitated 12 days after infection.

Results

KiSV-Induced Erythroid Leukemia. KiSV was rescued from a transformed nonproducer of NIH/3T3 cells with NB-tropic M-MuLV, N-tropic F-MuLV, or

N-tropic N35-MuLV and the titer of KiSV and helper virus were determined on NIH/3T3 cells. Virus stocks were inoculated into newborn Fv-1^{nn} CD-1 Swiss mice, a strain susceptible to KiSV-induced erythroid leukemia. 3–4 wk later, the animals were killed and their spleens were removed and examined to detect erythroid leukemia (5).

Unlike Friend virus (SFFV[F-MuLV]) (11), KiSV does not induce clearly defined foci of erythroid leukemia that can be counted grossly (5). To quantitate the leukemogenic activity of the KiSV, the incidence of erythroid leukemia was correlated with the number of FFU inoculated. It was found that approximately 10^2 FFU of KiSV(M-MuLV) or 10^3 FFU of KiSV(F-MuLV) induced erythroid leukemia in 50% of the mice (Fig. 1). As much as 10^5 FFU of KiSV(N35-MuLV), however, was required to induce leukemia in 50% of the mice, demonstrating that the induction of leukemia by the N35-MuLV pseudotype of KiSV was far less efficient. The helper viruses alone did not induce erythroid leukemia. The helper virus does, however, determine the efficiency of leukemogenesis induced by KiSV. This effect appears to be independent of the *Fv-1* locus, since the Fv-1^{nn} Swiss mice used in these experiments are susceptible to leukemogenesis induced by N-tropic KiSV(F-MuLV).

Recovery of KiSV from the Spleens of Leukemic and Non-Leukemic Mice. It is possible that the intraperitoneal inoculum of KiSV did not reach the spleens of the non-leukemic mice. To learn if KiSV was present in the spleens of these mice, the spleens of both leukemic and non-leukemic mice were assayed for the presence of virus. Replicating KiSV could be recovered from the spleens of both leukemic and non-leukemic animals inoculated with either KiSV(F-MuLV) or KiSV(N35-MuLV) (Table I), but not from mice infected with helper virus alone (5). Thus, the KiSV replicated in the spleen cells of the non-leukemic animals. Since spleens contain a heterogenous group of cells, it is possible that the erythroid precursors of non-leukemic animals were not infected with KiSV.

Although KiSV replicated in the spleens of the non-leukemic animals, the titer of KiSV and helper virus (F-MuLV or N35-MuLV) was approximately 100-fold higher in the spleens of the leukemic animals. This increased titer of virus in the leukemic mice probably reflects the replication of leukemia cells.

A-MuLV-Induced Lymphoid Leukemia. To learn if the helper viruses also influence A-MuLV-induced lymphoid leukemia, A-MuLV was rescued from an A-MuLV-transformed nonproducer of NIH/3T3 cells (6) with several NB-tropic (M-MuLV and R-MuLV) and N-tropic (F-MuLV, Ki-MuLV, and N35-MuLV) helper viruses. The virus stocks were titered on NIH/3T3 cells and inoculated into newborn Swiss mice. Inoculation with 1 × 10^3 FFU of A-MuLV(M-MuLV) induced Abelson leukemia in 47% of the mice within 6 wk, while inoculation with 1 × 10^2 FFU did not cause this disease (Table II). 42% of the mice inoculated with 5 × 10^3 FFU of A-MuLV(R-MuLV) and 17% of mice inoculated with 7 × 10^3 of A-MuLV(F-MuLV) developed Abelson leukemia within 6 wk. In contrast, mice inoculated with 1 × 10^4 FFU of A-MuLV(N35-MuLV), or 5 × 10^3 FFU of A-MuLV(Ki-MuLV) did not develop Abelson leukemia.

Inoculation of several of the helper viruses alone also induced leukemia, but these leukemias did not resemble Abelson disease. M-MuLV induced lymphoid thymic tumors after a latent period of 3 mo, while F-MuLV induced lymphoid

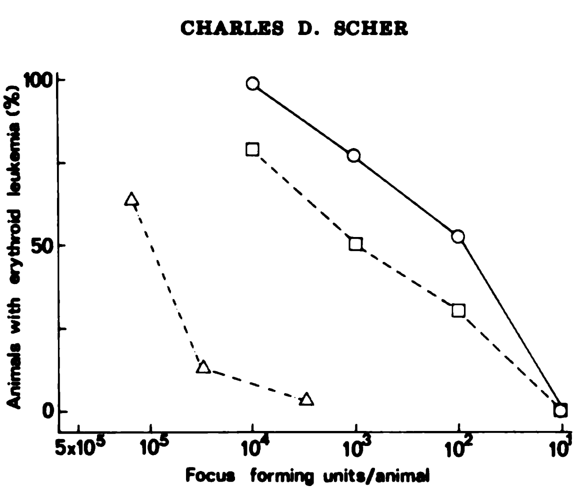

FIG. 1. The induction of erythroid leukemia by pseudotypes of KiSV. Virus stocks were titered and cell-free filtrates were inoculated into newborn Swiss mice. 3–4 wk later the animals were killed, touch preps of the spleens were made and analyzed histologically for evidence of erythroid leukemia. 20–30 animals were used for each dilution of virus stock. Legend: ○——○ KiSV(M-MuLV), 1 × 10^5 FFU/ml; 1 × 10^6 PFU/ml; □——□ KiSV(F-MuLV) 1 × 10^5 FFU/ml, 2 × 10^6 PFU/ml; △——△ KiSV(N35-MuLV) 3 × 10^6 FFU/ml, 1 × 10^7 PFU/ml.

TABLE I

*Recovery of KiSV from the Spleens of Infected Mice**

Virus inoculated	Erythroid‡ leukemia	Virus recovery	
		FFU	PFU
KiSV(F-MuLV)	Yes (3)	3 × 10^5–4 × 10^5	1 × 10^6
	No (2)	3 × 10^3–7 × 10^3	1 × 10^4–1 × 10^5
KiSV(N35-MuLV)	Yes (2)	4 × 10^4	1 × 10^5–6 × 10^5
	No (2)	5 × 10^2	1 × 10^3–4 × 10^3

* Newborn Swiss mice were inoculated with KiSV(F-MuLV) 3 × 10^3 FFU, 1 × 10^5 PFU, or KiSV(N35-MuLV) 2 × 10^5 FFU, 5 × 10^5 PFU. 4 wk later the animals were killed, and touch preps of the spleens were made for histological examination. The spleens were then shredded through a wire grid, a 10% (wt/vol) cell suspension was made, passed through a 0.45-μm membrane filter, and assayed on NIH/3T3 cells to determine the number of FFU of KiSV and PFU of helper virus per 0.5 ml of suspension.

‡ The number in parenthesis indicates the number of animals with or without erythroid leukemia whose spleens were removed and assayed.

splenic tumors detectable 6 wk or more after inoculation. The R-MuLV used in these experiments contained the SFFV component (16) and many of the mice developed erythroid leukemia detectable in the spleen as early as 3 wk after inoculation. N35-MuLV and Ki-MuLV did not induce leukemia during the period of observation. The presence of Abelson leukemia could be differentiated from all of these other leukemias both grossly and histologically. Mice with Abelson leukemia developed lymphoid tumors within 3–6 wk of inoculation which involved the bone marrow and/or peripheral lymph nodes. Involvement of the cranial bone marrow and meninges was characteristic.

TABLE II

The Effect of the Helper Virus on the Frequency of A-MuLV-Induced Lymphoid Leukemia in Fv-1^{nn} Swiss Mice

Virus*	FFU inoculated	Leukemic mice‡/ total mice	With leukemia
			%
A-MuLV(M-MuLV)	1×10^4	17/25	68
	1×10^3	8/17	47
	2×10^2	0/30	0
A-MuLV(R-MuLV)	5×10^3	8/19	42
A-MuLV(F-MuLV)	7×10^3	4/24	17
A-MuLV(Ki-MuLV)	5×10^3	0/14	0
A-MuLV(N35-MuLV)	1×10^4	0/12	0

* Virus titers were A-MuLV(M-MuLV) 4×10^4 FFU/ml, 2×10^5 PFU/ml; A-MuLV(R-MuLV) 5×10^4 FFU/ml, 3×10^5 PFU/ml; A-MuLV(F-MuLV) 7×10^4 FFU/ml, 2×10^5 PFU/ml; A-MuLV(Ki-MuLV) 5×10^4 FFU/ml, 2×10^4 PFU/ml; A-MuLV(N35-MuLV) 1×10^5 FFU/ml, 5×10^5 PFU/ml.

‡ Mice with Abelson leukemia/total number of mice.

DBA/2 mice, an Fv-1^{nn} inbred strain, are also susceptible to Abelson leukemia. Inoculation of 1×10^3 FFU of A-MuLV(M-MuLV) into newborn DBA/2 mice induced Abelson leukemia in 90% of the animals, and 7×10^3 FFU of A-MuLV(F-MuLV) caused Abelson leukemia in 51%. A-MuLV(Ki-MuLV) (5×10^3 FFU) or A-MuLV(N35-MuLV) (1×10^4 FFU) did not induce Abelson leukemia in these mice (Table III). The experiments with DBA/2 and Swiss mice demonstrate that the helper virus determines the incidence of A-MuLV-induced lymphoid leukemia.

Transformation of Bone Marrow Cells by A-MuLV. The pseudotype of A-MuLV may directly affect its ability to cause a leukemic change in the hematopoietic cells. To test this hypothesis bone marrow cells were infected with various pseudotypes of A-MuLV. Marrow was taken from C57/L mice, a Fv-1^{nn} inbred strain susceptible to infection by N- or NB-tropic viruses; the marrow cells of this strain can readily be transformed by A-MuLV(M-MuLV) (3). Cells were infected with pseudotypes of A-MuLV and plated in an agarose gel containing 5×10^{-5} M 2-mercaptoethanol. Large transformed lymphoid colonies, consisting of 1,000 or more cells, were identified 12–14 days later. These colonies were not noted in mock-infected cultures, or in cultures infected with the helper viruses alone.

Infection of marrow cells with 1.5×10^5 FFU of A-MuLV(M-MuLV) transformed 9.7 lymphoid colonies per plate, while infection with 1.5×10^4 FFU of A-MuLV(M-MuLV) transformed 1.5 colonies per plate. At either multiplicity, approximately 5–10 transformed lymphoid colonies were found per 10^5 FFU of A-MuLV(M-MuLV) added (Table IV). Infection with A-MuLV(R-MuLV) allowed the recognition of 6.5 transformed colonies per 10^5 FFU, while infection with A-MuLV(F-MuLV) caused the growth of four transformed colonies per 10^5

TABLE III

The Effect of the Helper Virus on the Frequency of A-MuLV-Induced Lymphoid Leukemia in Fv-1^{nn} *DBA/2 Mice*

Virus*	FFU inoculated	Leukemic mice/total mice	With leukemia
			%
A-MuLV(M-MuLV)	1×10^4	6/6	100
	1×10^3	9/10	90
	1×10^2	0/20	0
A-MuLV(F-MuLV)	7×10^3	21/41	51
A-MuLV(Ki-MuLV)	5×10^3	0/14	0
A-MuLV(N35-MuLV)	1×10^4	0/10	0

* Virus titers were A-MuLV(M-MuLV) 1×10^5 FFU/ml, 1×10^6 PFU/ml; A-MuLV (F-MuLV) 7×10^4 FFU/ml, 2×10^5 PFU/ml; A-MuLV (Ki-MuLV) 5×10^4 FFU/ml, 2×10^4 PFU/ml; A-MuLV (N35-MuLV) 1×10^5 FFU/ml, 5×10^6 PFU/ml.

TABLE IV

The Effect of the Helper Virus on the Frequency of A-MuLV-Induced Bone Marrow Cell Transformation in Fv-1^{nn} *C57/L Mice*

Virus*	FFU inoculated‡ per plate (× 10^5)	Transformed colonies§/total plates	Transformed colonies/plate	Transformed colonies/10^5 FFU
A-MuLV(M-MuLV)	1.5	97/10	9.7	6.5
	0.15	15/10	1.5	10
A-MuLV(R-MuLV)	1.0	65/10	6.5	6.5
A-MuLV(F-MuLV)	0.35	14/10	1.4	4.0
A-MuLV(Ki-MuLV)	0.30	0/13	0	0
A-MuLV(N35-MuLV)	0.50	1/21	0.04	0.1
None	0	0/20	0	0

* Virus titers were A-MuLV(M-MuLV) 3×10^5 FFU/ml, 2×10^6 PFU/ml; A-MuLV(R-MuLV), 2×10^5 FFU/ml, 6×10^5 PFU/ml; A-MuLV(F-MuLV) 7×10^4 FFU/ml, 2×10^5 PFU/ml; A-MuLV(Ki-MuLV) 4×10^4 FFU/ml, 2×10^4 PFU/ml; A-MuLV(N35-MuLV) 1×10^5 FFU/ml, 5×10^5 PFU/ml.

‡ Bone marrow cells (1.5×10^6) were inoculated with the FFU of virus shown, and after the adsorption period, were placed in a soft agarose gel in a single plate.

§ Transformed colonies of lymphoid cells were enumerated 12 days after the virus adsorption period.

FFU. A-MuLV(N35-MuLV) and A-MuLV(Ki-MuLV), however, transformed these bone marrow cells poorly. Approximately 0.1 transformed lymphoid colonies were found per 10^5 FFU of A-MuLV(N35-MuLV), while no colonies were detected after infection with A-MuLV(Ki-MuLV). Thus, the helper virus profoundly influences the ability of A-MuLV to induce a leukemic change in hematopoietic cells in vitro.

Complementation of A-MuLV-Induced Transformation by M-MuLV. To learn if M-MuLV complements A-MuLV to allow the transformation of hema-

TABLE V

The Effect of Added M-MuLV on the Frequency of Bone Marrow Cell Transformation by A-MuLV(N35-MuLV)

Virus*	FFU Inoculated per plate (× 10^5)	Addition of M-MuLV‡ (moi 3)	Transformed colonies/total plates	Transformed colonies/plate	Transformed colonies/10^5 FFU
A-MuLV(M-MuLV)	0.5	No	106/10	10.6	21.2
		Yes	30/10	3.0	6.0
A-MuLV(N35-MuLV)	0.5	No	1/10	0.1	0.2
		Yes	0/10	0	0
None	0	No	0/20	0	0
		Yes	0/20	0	0

* Virus titers determined on NIH/3T3 cells: A-MuLV(M-MuLV) 1 × 10^5 FFU/ml, 2 × 10^6 PFU/ml; A-MuLV(N35-MuLV) 1 × 10^5 FFU/ml, 1 × 10^5 PFU/ml; M-MuLV 1 × 10^7 PFU/ml.

topoietic cells, bone marrow cells were infected with poorly transforming A-MuLV(N35-MuLV) in the presence of excess M-MuLV (multiplicity of infection [moi 3]). This added M-MuLV did not increase the frequency of transformation induced by A-MuLV(N35-MuLV) (Table V). Similarly, M-MuLV did not increase the frequency of transformation induced by A-MuLV(M-MuLV). Thus, the pseudotype of A-MuLV, rather than the presence of a complementing effective helper virus, determines the lymphoid transformation frequency.

Isolation of an A-MuLV-Transformed Lymphoid Nonproducer. Four clones of lymphoid cells from C57/L mice were isolated from cultures infected with A-MuLV(M-MuLV) grown to mass culture and studied for the replication of A-MuLV and M-MuLV. Three of these clones released infectious A-MuLV(M-MuLV) while one (clone 3) released neither infectious A-MuLV nor M-MuLV. Media in which clone 3 was grown contained no detectable reverse transcriptase demonstrating that it was not producing virus particles. After infection with M-MuLV, clone 3 released infectious A-MuLV which transformed NIH/3T3 cells. This experiment demonstrates that the A-MuLV component of A-MuLV(M-MuLV) transformed the bone marrow cells.

Discussion

Several defective viruses induce leukemia after inoculation into mice. A-MuLV causes a null cell lymphoid leukemia (1, 4), while SFFV (11, 16) and KiSV (5) induce an erythroid leukemia. The helper viruses do not produce these types of leukemia. The present study shows that the helper virus determines the leukemogenic activity of A-MuLV for lymphoid cells and KiSV for erythroid cells.

The murine genetic locus, *Fv-1*, restricts the replication of competent helper viruses. Restriction is dominant and appears to act after virus absorption and penetration (17, 18). The pseudotype of a defective agent, including its N- or B-tropism, is determined by the helper virus (19). The *Fv-1* locus can restrict both the sarcoma virus-induced transformation of fibroblasts in vitro (19), and SFFV-induced erythroid leukemia in vivo (8). In the present study Fv-1^{nn} hosts were infected with N- or NB-tropic pseudotypes of defective viruses. The viruses were grown and titered on FV-1^{nn} NIH/3T3 cells. However, these virus stocks varied widely in their ability to induce leukemia. Clearly the Fv-1^{n} phenotype is not responsible for the observed restriction of leukemogenic activity.

There are two classes of murine ecotropic helper viruses. The endogenous viruses, typified by N35-MuLV, are integrated into the host's germ line, and are inherited in a vertical fashion. These viruses may begin to replicate in elderly mice and can often be induced to replicate in cells in vitro, after the addition of halogenated pyrimidines (20). Inoculation of endogenous viruses into susceptible mice may result in the formation of tumors, but these tumors usually apear at low frequency after a prolonged latent period that is often greater than 1 yr (21). The exogenous viruses, typified by F-MuLV, M-MuLV, and R-MuLV (FMR group), are usually not inherited vertically (22). Inoculation of helper FMR viruses into mice results in a high frequency of lymphoid tumors with a shorter latent period (1½–6 mo). The present study shows that an FMR-pseudotype of either A-MuLV or KiSV induces leukemia far more efficiently than an N35-MuLV endogenous virus pseudotype of these two defective transforming viruses. In addition, a Ki-MuLV pseudotype of A-MuLV does not induce Abelson leukemia. Ki-MuLV shares common envelope and p12 antigens with the endogenous viruses (13). Thus, mice appear to be able to restrict leukemia induced by endogenous viruses alone, and leukemia induced by an endogenous virus pseudotype of a defective transforming virus.

Virus spread may contribute to the induction of leukemia in vivo by either A-MuLV or KiSV. Certainly, the titer of KiSV present in the spleens of nonleukemic animals is lower than the titer in leukemic animals (Table I). In addition, an endogenous virus pseudotype of A-MuLV, that does not induce leukemia in vivo, cannot be recovered from the hematopoietic tissues of mice (23).

To study a single-step leukemia transformation event, I infected bone marrow cells with pseudotypes of A-MuLV and plated the cells in a soft agarose gel. Rosenberg and Baltimore (3) have demonstrated that the number of transformed lymphoid cells recognized in this gel culture system is directly proportional to the virus inoculum (one-hit kinetics). Thus, transformation is the result of a single-step event rather than being caused by virus spread.

The A-MuLV genome appears to be responsible for inducing leukemic transformation, because nonproducer cells have been isolated. These nonproducer lymphoid cells are isolated at a low frequency (1/4) because of the presence of excess helper virus in the inoculum. Murine sarcoma virus-transformed nonproducer BALB/c-3T3 cells have been isolated at a comparable frequency (24).

Although A-MuLV appears to contain the genetic information that controls lymphoid cell transformation, the present study shows that the helper virus also has an important role. FMR helpers allow a high frequency of transformation while the N35-MuLV endogenous virus or Ki-MuLV do not. Mixing experiments demonstrate that the addition of an effective helper such as M-MuLV to stocks of poorly transforming A-MuLV (N35-MuLV) does not enhance lymphoid transformation. Since complementation does not occur, the pseudotype of A-MuLV, rather than the presence of an exogenous effective helper, controls the transformation of hematopoietic cells.

The pseudotype of A-MuLV affects its ability to transform hematopoietic cells in vitro. It is likely that the pseudotype of A-MuLV and KiSV, rather than the presence of an effective complementing helper also controls leukemogenesis in vivo. This control of leukemogenesis by the helper virus may be a general one.

N35-MuLV and Ki-MuLV pseudotypes of A-MuLV and KiSV readily transform NIH/3T3 fibroblast-like cells. However, the virus-induced leukemic change in hematopoietic cells is restricted both in vivo and in vitro. The mechanism of restriction remains unclear. One possibility is that the hematopoietic cells block an early event in the infectious cycle.

Summary

Nonproducer cells transformed by Kirsten sarcoma virus (KiSV) or Abelson murine leukemia virus (A-MuLV) were infected with N- or NB-tropic helper viruses to rescue the defective transforming virus. The titer of the transforming viruses was determined on NIH/3T3 fibroblast-like cells and cell-free filtrates of virus stock were inoculated into newborn Fv-1^{nn} mice. Friend, Moloney, and Rauscher group of MuLV (FMR) pseudotypes of KiSV induced an erythroid leukemia efficiently, while an endogenous helper (N35-MuLV) pseudotype of KiSV did not. FMR pseudotypes of A-MuLV induced the Abelson lymphoid leukemia, while the N35-MuLV or a Kirsten leukemia virus (Ki-MuLV) pseudotype did not.

Pseudotypes of A-MuLV were used to infect bone marrow cells of Fv-1^{nn} mice in vitro. The FMR pseudotypes transformed bone marrow cells at 40–100-fold higher frequency than the N35-MuLV or Ki-MuLV pseudotypes. Mixing experiments demonstrated that the addition of an effective helper, such as M-MuLV, did not enhance lymphoid transformation by ineffective A-MuLV(N35-MuLV). The A-MuLV genome is responsible for hematopoietic cell transformation because a nonproducer clone of lymphoid cells, free of helper virus, was isolated. The data indicates that the pseudotype of A-MuLV determines its ability to transform hematopoietic cells.

I thank Doctors G. Cooper and W. Haseltine for review of the manuscript and Miss Stone, Miss J. Fontaine, and Mr. I. Rosenberg for able technical assistance.

Received for publication 4 October 1977.

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DISTRIBUTION OF A MAJOR CONNECTIVE TISSUE PROTEIN, FIBRONECTIN, IN NORMAL HUMAN TISSUES*

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Fibronectin (1) is a polymorphic glycoprotein found in blood and tissues of vertebrates (2) and in cultures of adherent fibroblastic (3) and astroglial (4) cells. The circulating form of this glycoprotein was described nearly 30 years ago as "cold-insoluble globulin" which co-precipitates with fibrinogen in the cold (5). The concentration in normal human plasma is about 300 μg/ml (6). Fibronectin is a major protein in cultures of fibroblastic cells (3) from various species, and has also been known as LETS protein or CSP (7-11). Fibronectin of cultured human fibroblasts and that of human plasma are immunologically identical and have the same mobility in immunoelectrophoresis (3, 12). Both are dimers of disulfide-bonded 200,000-220,000 mol wt polypeptides (13-15).

The biological role of fibronectin is poorly understood. The protein has been studied mostly in cell culture conditions, where it seems to be involved in cell adhesion (16-18). In chicken and mouse embryos, fibronectin is found in basement membranes and in the extracellular connective tissue matrix (19, 20). In the present work, immunofluorescence has been used to demonstrate fibronectin in normal human adult tissues.

Materials and Methods

Anti-Fibronectin Serum. Antiserum to human plasma fibronectin (cold-insoluble globulin) was raised in rabbits. The purity of the antigen used for immunization was controlled with sodium dodecyl sulphate-polyacrylamide gel electrophoresis, in which a single polypeptide band was detected. Immunofluorescence obtained in indirect staining was completely blocked by preincubation of the antiserum with purified plasma fibronectin. The anti-fibronectin serum gave a single precipitation line against normal human plasma, against extract of human fibroblasts, and against the purified antigen in immunodiffusion analysis (17), and it gave a single polypeptide band in immunoprecipitation with radiolabeled fibroblast extracts (16), as was documented previously.

Histologic Techniques. All tissue specimens were obtained as fresh surgical biopsy samples. For immunofluorescence staining, we used frozen sections fixed in acetone (−20°C, 20 min) or paraffin-embedded (56°C, the method described in ref. 21) sections fixed in alcohol.

Conventional histological techniques (22) were used to demonstrate collagen (Van Gieson's stain), elastin (Weigert's resorcin-fuchsin elastic stain) and reticulin (Gomori's reticulin stain). Sections previously stained for immunofluorescence were fixed in 3.5% formaldehyde before histologic staining.

Immunofluorescence Staining. Fixed sections were washed twice for 10 min in phosphate-buffered saline (PBS),[1] and treated for 30 min at room temperature with rabbit anti-fibronectin serum diluted 1:40 in PBS. The PBS washes were repeated, and fluorescein isothiocyanate (FITC)-conjugated sheep anti-rabbit gamma globulin (Wellcome Research Laboratories, Beckenham, England) diluted 1:40 in PBS was soaked on the sections for 30 min at room temperature. After two 10-min washes with PBS, the preparation was mounted using a solution containing equal volumes of glycerol and Veronal-buffered (50 mM, pH 8.6) sodium chloride (100 mM).

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* Supported by grant CA 17373 of the National Cancer Institute, and by grants from the Sigrid Jusélius Foundation and the President J. K. Paasikivi Foundation, Helsinki, Finland.

[1] *Abbreviations used in this paper:* PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate.

In control experiments, the anti-fibronectin rabbit serum was substituted either with normal rabbit serum, with anti-fibronectin serum pretreated with purified plasma fibronectin (50 μg/ml), or with PBS.

Fluorescence Microscopy. Stained sections were examined with a Zeiss Universal microscope equipped with the epi-illuminator III RS (Carl Zeiss, Oberkochen, West Germany) and an HBO 200 W high pressure mercury lamp. The filters used for specific FITC fluorescence were excitation filters BP 455–490, dichroic mirror FT 510, and barrier filters LP 520. All sections were tested for autofluorescence by using the intense green emission light (545 nm) of the mercury lamp for fluorescence excitation with excitation filter BP 546/10, dichroic mirror FT 580, and barrier filter LP 590. Autofluorescence was then intensely red and could be distinguished from the green FITC fluorescence which was detected with a blue emission light. Only those specimens without autofluorescence were studied, except for detection of autofluorescing elastic laminae in arteries (e.g. Fig. 4b).

Photographs were made on Agfapan 400 Professional film developed in Atomal FF (Agfa-Gaevert, Leverkusen, West Germany) to normal contrast at a sensitivity of 26 DIN.

Results

General Findings. Fibronectin was ubiquitously present in all studied tissues. It was especially abundant in basement membranes, around smooth muscle cells and striated muscle fibers, in the connective tissue stroma of lymph nodes and the spleen, and in sinusoidal walls of the liver. The fluorescence was specific, as control-stained sections were negative (see Materials and Methods), and autofluorescence was absent or very weak.

In all sites, the distribution of fibronectin corresponded to histologically demonstrated reticulin (Fig. 1), but not to collagen or elastic fibers. In several sites, however, reticulin and collagen were present in the same structures. By sequentially using polarization and fluorescence microscopy, it could be demonstrated that birefringent collagen bundles and fluorescing fibronectin fibers were separate structures, e.g. in the spleen trabeculae and capsule and dermis. The collagen bundles and fibronectin fibers were often situated close together.

Lymphatic Tissues. The spleen (Fig. 2) contained fibronectin in a dense network which branched throughout the tissue. The network was sparse or absent in germinal centers. Thin fibers of fibronectin were packed between collagen bundles in the capsule and trabeculae. In the lymph nodes (Fig. 3), a similar but more sparse network was seen. Little or no fibronectin was detected in the germinal centers. Sinusoidal walls and the capsule contained a dense, continuous fibronectin stroma.

Blood Vessels. All blood vessels, including capillaries, contained a zone of fibronectin corresponding to the basement membrane of the endothelium (Fig. 4). The internal elastic lamina, which contains elastic fibers (in many specimens autofluorescing), was outside this zone and clearly separated from it (compare Figs. 4a and b). Fibronectin was also present in the loose connective tissue of the adventitia. In muscular arteries (Fig. 4a), fibronectin was further found around the smooth muscle cells of the media. In nonmuscular arteries (Fig. 4c), the media contained thin strands of fibronectin.

Muscle. Striated muscle fibers contained fibronectin in the sarcolemma,

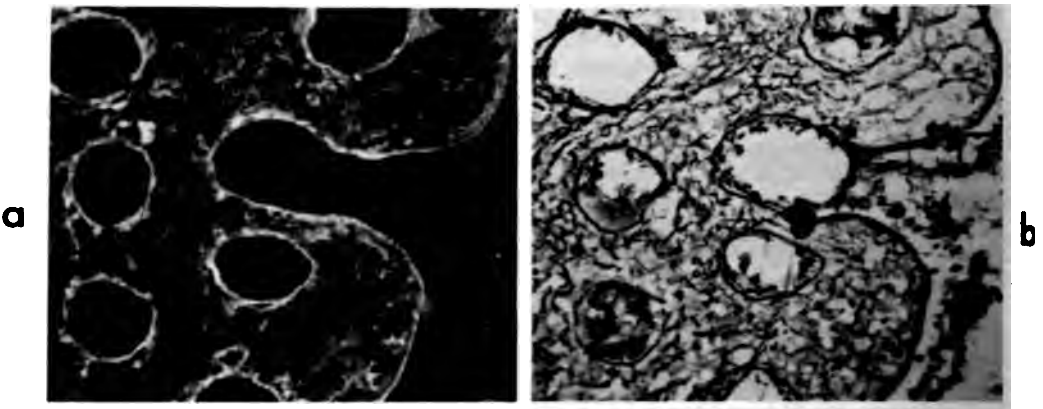


FIG. 1. Immunofluorescence staining for fibronectin (a) and histological staining for reticulin (b) of the same section of large intestine mucosa. Fibronectin is seen in basement membranes and in the loose connective tissue of the lamina propria. × 400.

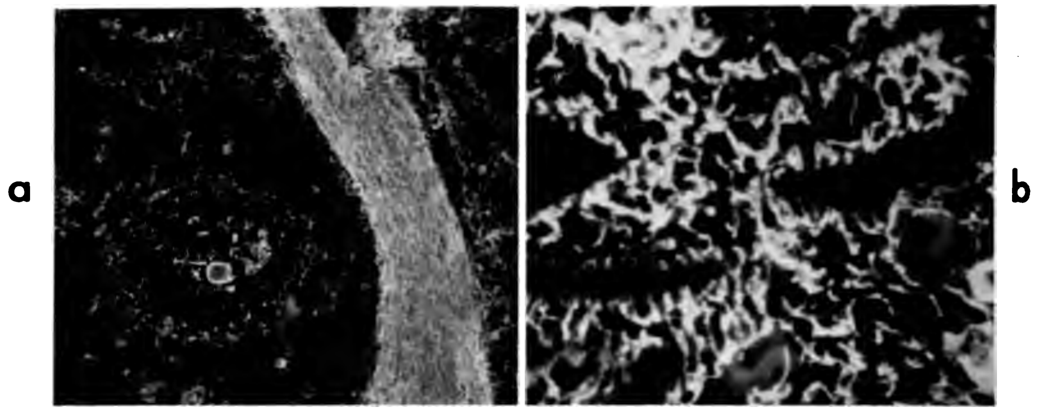


FIG. 2. Spleen. Fibronectin is abundant in trabeculae (a), and in the stroma a dense fibronectin-containing network is visible (b). (a), × 65; (b), × 400.

where a continuous sheet was visualized (Fig. 5). In smooth muscle, fibronectin was present in the pericellular coating of individual cells (Fig. 6).

Respiratory Tract. In the trachea and bronchi, fibronectin was seen as a zone corresponding to the basement membrane beneath the epithelium. In the alveolar walls (Fig. 7), fibronectin was seen around the capillaries and at sites corresponding to the basement membrane of the alveolar epithelium.

Glands. The basement membranes in glands contained fibronectin. This was especially conspicuous in the thyroid gland (Fig. 8). In the mammary gland (Fig. 9), the fibronectin-specific fluorescence of the basement membranes was weaker. In the parathyroid gland (Fig. 10), little fibronectin was detected outside intensely staining blood vessels.

Liver. The sinusoids of the liver had a continuous thin zone of fibronectin probably located between the sinusoidal lining and liver cell plates (Fig. 11). No fibronectin was visualized between hepatocytes. In portal areas, fibronectin was present in the loose connective tissue and in blood vessels.

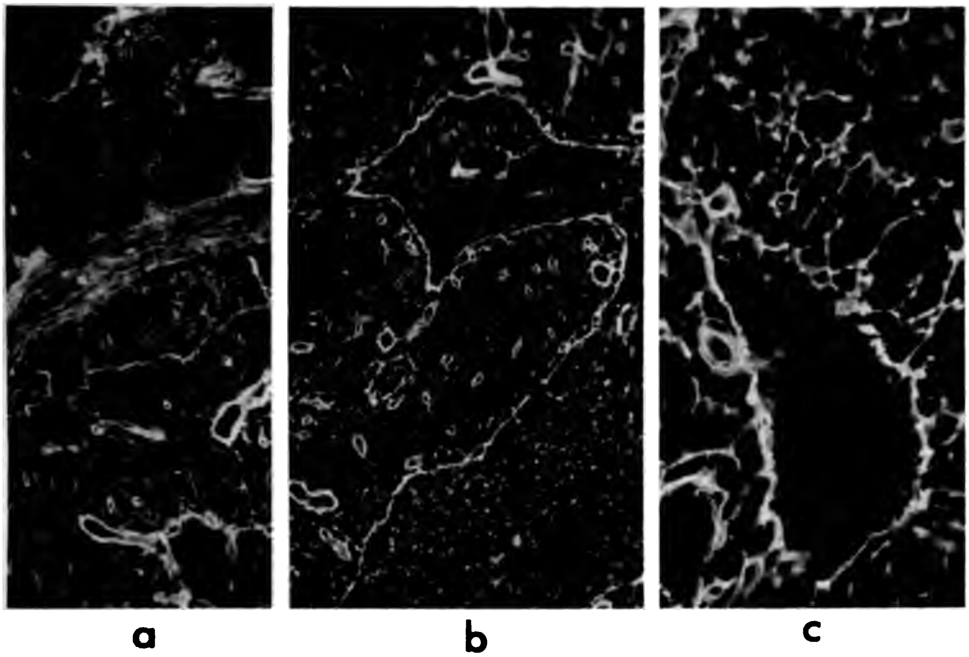


Fig. 3. Lymph node. (a) The capsule contains fibronectin and the surface of individual fat cells outside the capsule is also positive in immunofluorescence staining. In the stroma a loose fibronectin-containing network is observed. Fibronectin forms a continuous sheet in the surface of sinusoid walls (b) and capillaries (c). (a) and (b), × 130; (c), × 320.

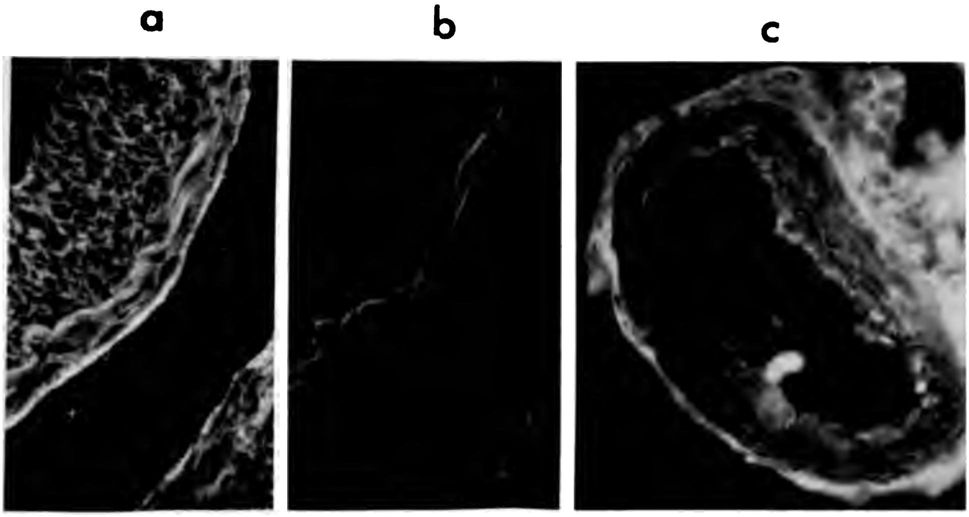
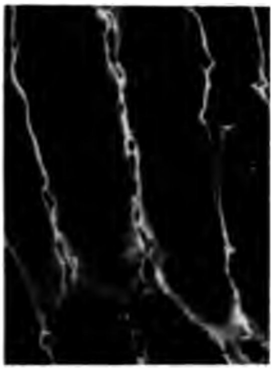


Fig. 4. Artery. (a) The basement membrane of the endothelium contains fibronectin, and individual muscle cells show fibronectin in the cellular coating. The internal elastic lamina was autofluorescent in this specimen. It is shown separately in (b) clearly separated from the endothelial basement membrane. In specimens without autofluorescence, the elastic lamina is not visualized and does not contain fibronectin. In nonmuscular arteries (c), the basement membrane contains fibronectin. Thin strands of fibronectin are also seen in the media and large amounts of fibronectin are visible in the adventitia. × 320.



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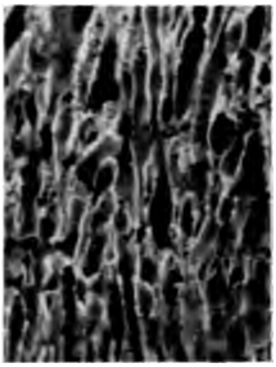

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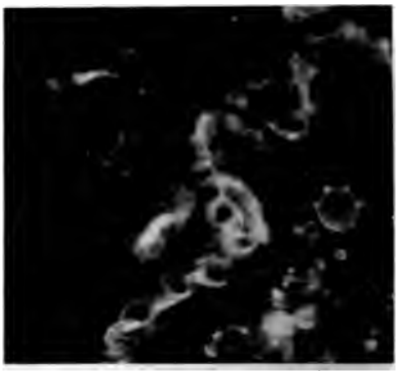

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FIG. 5. Striated muscle. Fibronectin is located in the sarcolemma. × 320.

FIG. 6. Smooth muscle of the large intestine. The pericellular coating is rich in fibronectin. Smooth muscle cells in other sites were similarly stained (see Fig. 4 a). × 400.

FIG. 7. Lung. The alveolar basement membrane contains fibronectin. × 400.

Gastrointestinal Tract. In the stomach (Fig. 12), fibronectin was distinctly distributed in the region of the basement membrane of the surface epithelium. In the muscular layer, fibronectin was present around the individual smooth muscle cells (Fig. 6). In the lamina propria, fibronectin occurred as thin strands of the loose connective tissue and in blood vessel walls as described above. The basement membrane of the mesothelium was rich in fibronectin. In the intestine (Fig. 1 a), fibronectin was distributed in the same manner as that in the stomach.

Kidney and Urogenital Tract. Glomeruli contained fibronectin in sites corresponding to the mesangial cells (Fig. 13). Fibronectin was also detected around the tubuli at sites probably corresponding to capillaries and the interstitial connective tissue (Fig. 14). In testis (Fig. 15) and epididymis (Fig. 16), basement membranes and loose connective tissue of the stroma contained fibronectin.

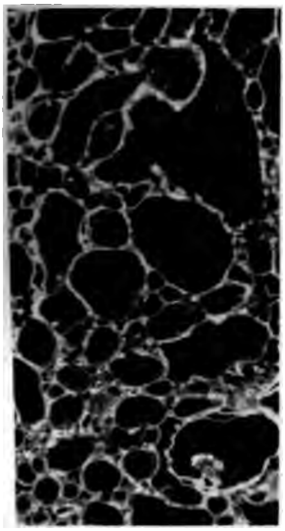
Skin. The dermal connective tissue contained thin strands of fibronectin (Fig. 17) intercalated with abundant collagen bundles. Some fibronectin was detected in the basement membrane of the epidermis.

Connective Tissue. Fibronectin was characteristically present in loose connective tissue around blood vessels (Fig. 4 c) and nerves and in the lamina propria of the gastrointestinal tract (Figs. 1 a and 12). In the connective tissue of the mammary gland only thin fibronectin strands occurred in the collagen-rich stroma, but around ducts and lobuli, fibronectin formed a sparse network (Fig. 9). A similar distribution of thin fibronectin fibers was observed in the collagen-rich dermis (Fig. 17). In the loose connective tissue of testis and epididymis, fibronectin was predominant (Figs. 15 and 16). In fat tissue (Fig. 3 a), fibronectin was seen on the surface of individual fat cells. In basement membranes, fibronectin was abundant, except in the skin and kidney.

Discussion

This work shows that fibronectin is a major connective tissue protein in adult

8



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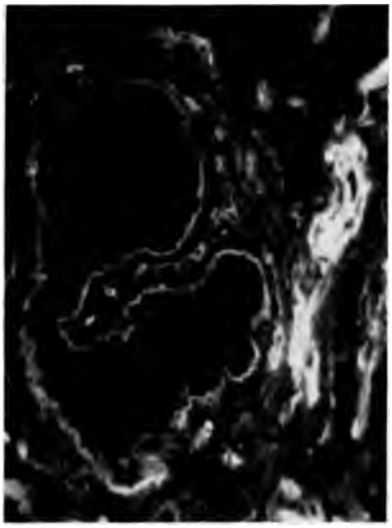

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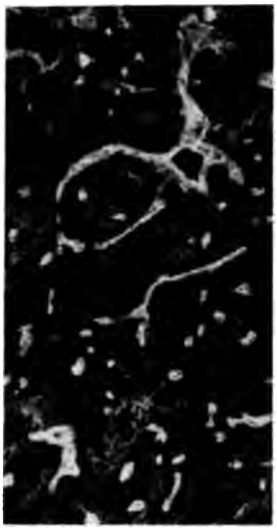

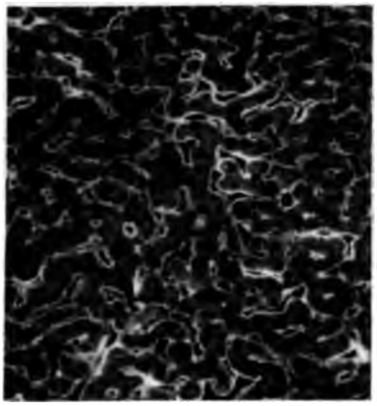

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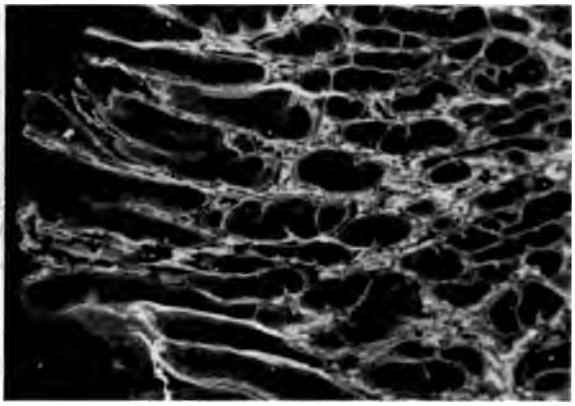

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Fig. 8. Thyroid gland. Fibronectin appears in basement membranes. × 50.

Fig. 9. Mammary gland. Fibronectin in basement membranes and in the connective tissue appears as thin strands. Large amounts of fibronectin are seen in the wall of a small vein to the right of the fig. × 160.

Fig. 10. Parathyroid gland. Fibronectin is present in capillary walls only. × 65.

Fig. 11. Liver. Fibronectin is located in the perivascular tissue of sinusoids. × 130.

Fig. 12. Stomach. Fibronectin can be seen in basement membranes of the surface epithelium and in the loose connective tissue of the lamina propria. The basement membrane of the mesothelium also contained fibronectin (not shown). × 50.

human tissues, typically occurring in most basement membranes, in the reticulin stroma of lymphatic tissues, and in loose connective tissue. In smooth and striated muscle cells, fibronectin is located in the pericellular coating of individual cells. In all these sites where fibronectin was detected by immunofluorescence, reticulin could be demonstrated with histological staining, but no codistribution of fibronectin with collagen and elastic fibers was observed.

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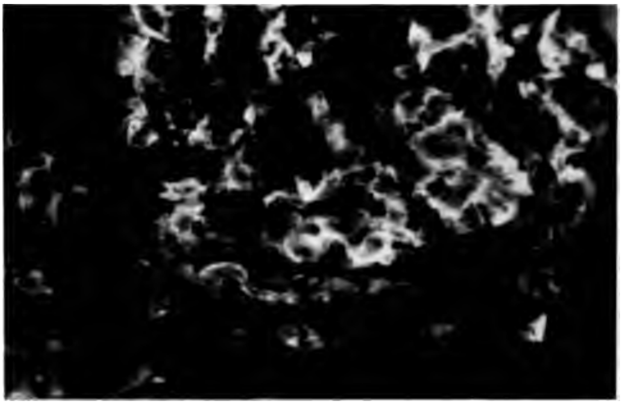

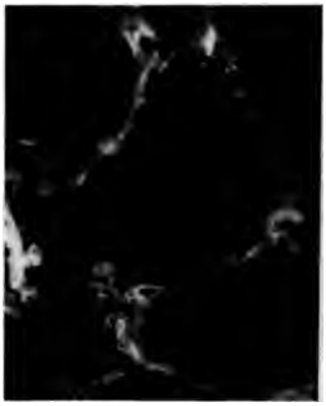

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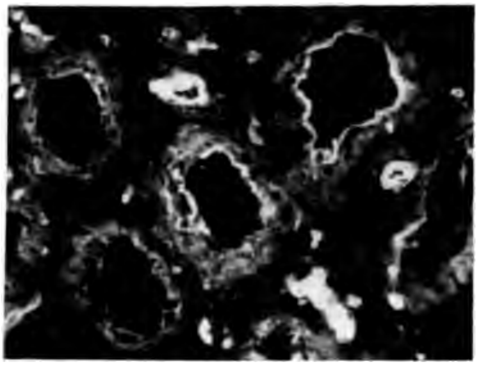

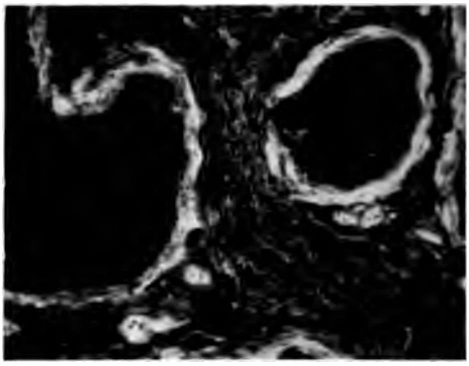

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FIG. 13. Glomerulus of the kidney. Fibronectin is distributed in the mesangium but not in basement membranes. × 400.

FIG. 14. Kidney tubuli. Fibronectin occurs in interstitial tissues and around capillaries, but not in basement membranes of tubuli. × 400.

FIG. 15. Testis. Fibronectin is visible in basement membranes and in loose connective tissue stroma and blood vessel walls. × 160.

FIG. 16. Epididymis. Fibronectin strands are seen in basement membranes and loose connective tissue. × 160.

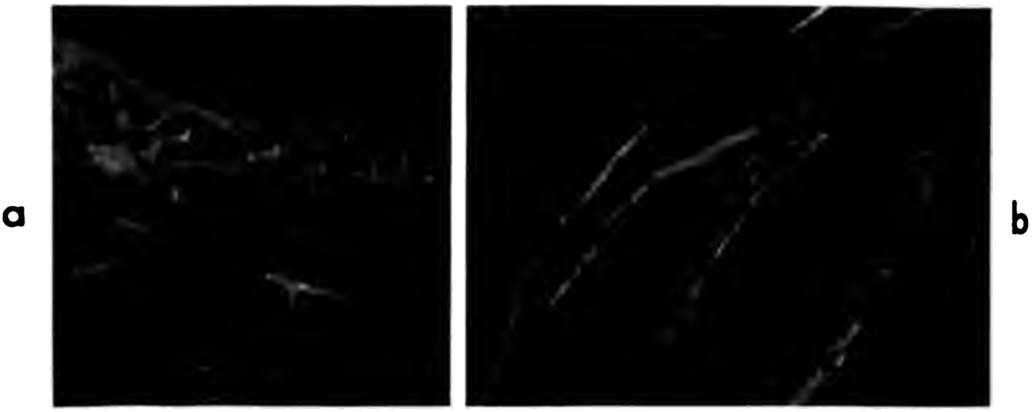

FIG. 17. Skin. (a) In the papillary connective tissue a fine network of fibronectin can be seen (bottom of fig.). The dermo-epidermal junction is weakly positive and epidermis negative (top of fig.). (b) In the deep dermal connective tissue, fibronectin is seen as thin strands between fluorescence-negative collagen bundles. (a), × 400; (b), × 160.

Fibronectin is therefore one of the noncollagenous glycoproteins of connective tissue (23) and is obviously a component of reticulin, which is the descriptive name for fibers blackened by silver impregnation (22). Other proteins present in reticulin fibers include at least collagen type III (24) and some noncollagenous glycoproteins (25, 26). In mouse serum, a glycoprotein which chemically resembles fibronectin has been shown by immunohistological methods to cross-react with a protein present in reticulin fibers (27).

Most previous studies on the function of fibronectin have been done with cultivated fibroblasts, in which fibronectin is a major surface-associated glycoprotein. In fibroblast layers, fibronectin forms an extensive pericellular matrix which mediates cell-cell and cell-substratum contacts (3, 12). This matrix can be considered analogous to the fibronectin deposited in basement membranes. We therefore propose a structural role of fibronectin in positioning and anchoring cells in vivo. A similar function of fibronectin in cell adhesion in vitro has been proposed earlier (16–18).

The known biochemical interactions of fibronectin support the above hypothesis. Soluble fibronectin has a high affinity in vitro for collagenous proteins (28, 29), it interacts with the glycosaminoglycan heparin (30), and both soluble (11) and cell-associated (9) fibronectin are cross-linked to fibrinogen or fibrin by the action of plasma transglutaminase (activated blood coagulation factor XIII). In this way, fibronectin may in vivo form a structural link between the cell surface and collagenous proteins in the connective tissue matrix.

It is not clear whether the fibronectin of basement membranes is produced by connective tissue cells or by the adjacent epithelial cells. Data from in vitro experiments support both possibilities. First, fibroblasts produce in vitro a dense surface-associated matrix (3, 12). Second, undifferentiated cells from primitive mouse kidney mesenchyme produce in vitro a loose fibronectin network around individual mesenchymal cells. When this tissue is induced to differentiate into kidney tubuli, the fibronectin is confined to the basement membrane which is formed around tubuli (20). Similarly, when myoblasts fuse in vitro to form myotubes, fibrillar surface fibronectin is redistributed into a diffuse cell coating (31) analogous to that described here for muscle fibers. These data indicated that changes in fibronectin expression are closely linked with cell differentiation and organogenesis.

In cultures of fibroblastic (3) and astroglial (4) cells, fibronectin is detected intracellularly as a cell surface-associated matrix protein, and also secreted in large quantities into the culture medium. In the present in vivo study no intracellular fibronectin was detected, and no association between single connective tissue cells and fibronectin could be observed with certainty. The abundance of intracellular fibronectin and production of the protein into a pericellular matrix (16) in vitro may therefore be due to cell culture conditions allowing cell adhesion to a substratum and stimulation of cell proliferation.

Analogous situations in vivo would be chronic inflammation and formation of granulation tissue in wound healing, where fibroblasts characteristically proliferate. Our preliminary data from studies of such tissues show that, in fact, large amounts of fibronectin are detected in and around the proliferating fibroblasts. In such areas of wound healing, interaction of cellular or plasma (serum) (12) fibronectin with fibrin, collagen, and cell surfaces provides a link between proliferating cells and the connective tissue matrix around the damaged area or the clot formed within it. Similarly, fibronectin could play a role in the orientation of regenerating epithelial cells which proliferate on basement membranes (32).

Summary

Fibronectin is a major surface-associated glycoprotein of cultured fibroblasts and it is also present in human plasma. Antiserum specific for human fibronectin was used to study the distribution of fibronectin in normal adult human tissues. The protein was detected (*a*) characteristically in various basement membranes including capillary walls; (*b*) around individual smooth muscle cells and in the sarcolemma of striated muscle fibers; and (*c*) in the stroma of lymphatic tissue and as thin fibers in loose connective tissue. The distribution of fibronectin was distinct from that of collagen and elastic fibers, but was very similar to reticulin, as demonstrated by conventional histological staining. The results indicate that fibronectin is a major component of connective tissue matrix. The distribution also indicates that most types of adherent cells abut fibronectin-containing structures. This supports the possible role of fibronectin in cell-cell and cell-matrix interactions in tissues.

We thank Doctors Ewert Linder, Veli-Pekka Lehto, and Klaus Hedman for their critical evaluation of the manuscript, and Ms. Hannele Laaksonen, Ms. Ritva Nurme-Helminen, and Ms. Liisa Pitkänen for their technical assistance.

Received for publication 5 December 1977.

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T CELLS SPECIFIC FOR HAPTEN-MODIFIED SELF ARE PRECOMMITTED FOR SELF MAJOR HISTOCOMPATIBILITY COMPLEX ANTIGENS BEFORE ENCOUNTER WITH THE HAPTEN*

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One of the most striking developments in immunology in recent years has been the emergence of the concept that thymus-derived lymphocytes (T cells) recognize foreign, non-major histocompatibility complex (MHC) antigens in association with self MHC antigens. Several models have been proposed to account for these findings (1-5). There are basically two types of theories. One states that T cells have a single receptor with which they recognize a modification of self MHC antigens induced by the foreign antigen ("altered self"). The other states that T cells have two receptors or receptor sites, which must act in concert in order that activation occur, one receptor being specific for self MHC antigens and the other specific for the foreign antigen. At present, it is not possible to discriminate between these two possibilities. Indeed, inasmuch as most studies of MHC restriction have involved primed cells performing some effector function, it is not yet possible to state whether MHC restriction exists in T cells before their encounter with antigen, or whether it follows upon antigenic stimulation.

One approach to the problem of MHC restriction that has proven to be very valuable is T-cell cytotoxicity to 2,4,6-trinitrophenyl (TNP)-modified self target cells (5). In this model, it has been shown that cells from F_1 mice, immunized with TNP-modified parental cells, preferentially kill TNP-modified targets derived from the immunizing parental strain, as compared with TNP-modified targets from the other parent. The present experiments were initially undertaken to answer the question of whether F_1 mice contain a single set of precursors for TNP-reactive killer cells, which become committed to killing TNP-modified cells of one parent only after immunization, or, alternatively, have two sets of precursors, each already committed to recognize TNP-modified cells of one or the other parental MHC type. To study the state of commitment of the cells before immunization, the technique of antigen-driven, 5-bromodeoxyuridine (BUdR) and light suicide was used (6–8). By this means, F_1 cells responding to TNP-modified parental cells of one type could be eliminated, and the residual cells restimulated to see whether they retained the ability to generate cytotoxic effectors specific for TNP-modified cells from the other parental strain. These experiments gave results consistent with the recent studies of Zinkernagel et al. (9, 10) and of Bevan (11) in showing that F_1 T cells are precommitted to recognize TNP coupled to cells of one or the other parental strain.

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* Supported in part by U. S. Public Health Service grants AI 14579 and AI 13766. The work was begun while C. A. Janeway was a Moseley Traveling Fellow of Harvard Medical School and supported by grants from the Swedish Cancer Society.

‡ Investigator, Howard Hughes Medical Institute.

In carrying out these experiments, two other questions could readily be asked. The first involved the nature of the precursor of killer T cells capable of killing TNP-modified self cells, but which are induced by stimulation with allogeneic cells (5, 12). Such cells have previously been shown to differ from TNP-self killers induced by TNP-self stimulating cells in their specificity, a result we have confirmed. We can also state that such cells arise from different precursors than cells responding to TNP-self stimulators, because they are not affected by BUdR and light suicide with TNP-parental stimulators, under conditions where the response to the TNP-parental stimulator is totally eliminated.

The second question involved testing whether cells acutely depleted of alloreactive cells by BUdR and light suicide could respond to TNP-modified allogeneic cells of the type used to induce suicide. Although several authors have found such responses with acute depletion techniques (13, 14), our results strongly suggest that precursors for TNP-modified allogeneic cells are rare in normal mice. This result is again consistent with the elegant studies on virus-specific cytotoxic cells recently reported by Zinkernagel et al. (9, 10).

Materials and Methods

Mice. Mice of the strains CBA/H, A.CA, and (CBA/H × A.CA)F_1 were bred at the University of Uppsala (Uppsala, Sweden). Mice of the strains CBA/J, BALB/c, C57BL/6J (B6), DBA/2, A.SW, AKR/J, and (AKR × DBA/2)F_1 were purchased from The Jackson Laboratory, Bar Harbor, Maine. Mice of the strains A.TH, A.TL, and (A.TH × A.TL)F_1 were bred at Yale University (New Haven, Conn.). These mice were a generous gift of Dr. R. K. Gershon. The MHC haplotypes of these strains are given in Table I.

In Vitro Cultures. Spleen cell suspensions were prepared in Dulbecco's modified phosphate-buffered saline (PBS-D) by flushing them from the spleen with PBS-D with a syringe and needle. The red cells were lysed by brief (3 s) hypotonic shock, and the cells were then washed and counted in a hemacytometer; greater than 95% were viable by trypan blue dye exclusion. They were cultured in RPMI-1640 (Grand Island Biological Co., Grand Island, N.Y.) to which was added 10 ml 200 mM L-glutamine, 10 ml 1 M Hepes, 10 ml 7.5% $NaHCO_3$, 20 ml penicillin-streptomycin, 0.5 ml 0.1 M 2-mercaptoethanol, and 50 ml heat-inactivated fetal calf serum per liter. 30×10^6 responder cells were mixed with 30×10^6 allogeneic or 45×10^6 TNP-modified stimulator cells, which had been inactivated with 2,000 rads X-irradiation, in a volume of 14 ml medium in upright 50-ml tissue culture flasks (Falcon 3013, Falcon Plastics, Div. BioQuest, Oxnard, Calif.). The cultures were carried out in an atmosphere of 5% CO_2 in air at 37°C. These conditions were found to be optimal for these responses, and reproducibly led to nearly complete suicide of precursors. TNP modification of stimulator cells was performed as described by Shearer (5).

BUdR and Light Suicide. Preliminary experiments showed that the timing of addition of BUdR and time of lighting were critical for complete suicide (Table II). From this, we have evolved the following procedure, based on experience and subsequent experiments (8): 27 h after initiating the cultures, freshly prepared BUdR (ICN Pharmaceuticals, Cleveland, Ohio) is added to the cells and mixed to achieve a final concentration of 3×10^{-6} M. After 69 h of culture, the

TABLE I

MHC Haplotypes of the Mice Used in These Experiments

Strain	Haplotype of origin of MHC regions			
	K	*I*	*S*	*D*
A.CA	*f*	*f*	*f*	*f*
CBA/H, CBA/J, AKR/J	*k*	*k*	*k*	*k*
DBA/2, BALB/c	*d*	*d*	*d*	*d*
C57B1/6J (B6)	*b*	*b*	*b*	*b*
A.SW	*s*	*s*	*s*	*s*
A.TH	*s*	*s*	*s*	*d*
A.TL	*s*	*k*	*k*	*d*

TABLE II

Timing of BUdR and Light Is Critical in Suiciding TNP-Self Killer Cells

Time of adding BUdR (3×10^{-6} M)	% Specific ^{51}Cr release from TNP-CBA/H targets of cultures lit		
	48 h	72 h	96 h
h			
24	19	−5	26
48	–	2	23
72	–	–	27

CBA/H spleen cells were stimulated with TNP-CBA/H cells for 5 days and then assayed for cytotoxic activity on CBA/H and TNP-CBA/H targets as an effector: target ratio of 20:1; no release was seen on CBA/H targets. Untreated cultures gave 46% specific release on TNP-CBA/H targets. BUdR was added to various cultures at the times stated, and the cultures were exposed to light for 90 min at the stated times. Underlined value gives the greatest suicide.

flasks are removed from the incubator, the caps closed to prevent pH change, and the flasks placed upright over a fluorescent tube, separated from it by the thin portion of a Pasteur pipette, taking care not to disturb the cells. Lighting is for 90 min. The caps are then loosened, and the flasks returned to the incubator. After another 24 h, the cells are placed over the light a second time. They are then recovered from the flasks, pooled, washed three times, counted, and recultured with fresh stimulator cells. As a positive control, fresh cells are also cultured at this time. Finally, to control for suppressive effects, suicided cells are mixed 1:1 with fresh, syngeneic cells and cultured with the suiciding stimulator (or related stimulators). After a further 4 days in culture, the cells are recovered from the flasks, washed once, and assayed for cytotoxic activity on a variety of targets.

Cytotoxicity Assay and Calculation of Data. The cytotoxicity assay is a modification of that described by Simpson et al. (15). Targets are either spleen cells or peritoneal exudate cells induced by injection of 3 ml of 3% thioglycolate broth 3 days before use. Attackers are adjusted to identical counts of viable cells before assay. Some titrations are carried out, but as these only confirm the findings with undiluted cells, they have not been reported. The medium we use is RPMI-1640 with 10 mM Hepes, 0.075% $NaHCO_3$, and 5% heat-inactivated fetal calf serum. The assays are carried out in "V bottom" microtiter trays. Half the supernate is recovered 3.5 h later after centrifuging the plates and counted in a gamma counter, (LKB ultrogamma II, LKB Instruments, Inc., Rockville, Md.). The percent-specific ^{51}Cr release is calculated using the formula:

$$\%\ \text{Specific}\ ^{51}\text{Cr release} = \frac{\text{experimental counts} - \text{control (medium or normal cells) count}}{\text{total counts} - \text{control counts}}$$

Total counts are obtained from samples lysed under identical conditions with 1% Triton X-100, and spontaneous release values of 20-30% are usual. Standard errors, which are not reported, are generally <1-2%.

Results

The general scheme of the experiments is outlined in Fig. 1. F_1 spleen cells are stimulated either with TNP-modified parental or allogeneic cells, and cells responding to the initial stimulus are suicided with BUdR and light. This treatment removes both cytotoxic effector cells (Table II) and their precursors (Tables III-VII). The remaining cells are then cultured with various stimulator cells, and each group is assayed for cytotoxicity on a variety of target cell types. Each table in this paper represents results of a single experiment. To simplify the presentation of the results, each line is numbered, and the experiments are discussed according to the questions being asked, rather than one experiment at a time. Responses to unmodified, syngeneic, or parental targets were generally less than background lysis, and have been omitted. On rare occasions, lysis of unmodified targets was significantly above background, in which case they were subtracted from the release obtained with TNP-modified targets of the same type. This response to unmodified self or parental cells is probably analogous to that reported by Peck et al. (16), and was seen only with freshly cultured cells when stimulated by allogeneic cells. Its absence in all the groups in which precultured (and suicided) cells were used probably means that cells killing unmodified targets are eliminated in the primary, suiciding cultures.

The Specificity of Cytotoxic Effector Cells for TNP-Modified Targets. As has been shown by Shearer et al. (5), spleen cells stimulated with TNP-autologous cells will kill preferentially TNP-modified targets identical at the *K* and *D* loci of the MHC to the original stimulator and responder cell combination. This is seen throughout these experiments (Tables II-IX). Furthermore, F_1 cells stimulated with TNP-modified parental cells lyse TNP-modified targets of the original immunizing parental strain preferentially, with two exceptions (Table VI, line 36; Table VII). The former exception was found only in this one experiment and is unexplained, whereas the latter demonstrates that when the parental strains are identical at the *K* and *D* loci, even if they differ throughout the *I* region of the MHC, then killers induced by TNP-modified cells of either parental strain will kill TNP-modified targets of either parental type. This finding is consistent with Shearer's previous results, and strongly suggests that killing directed at TNP-modified *I* region determinants does not play a significant role in our experiments.

The experiment reported in Table VIII demonstrates that although T cells stimulated with TNP-self cells will kill TNP-self cells, they will not kill allogeneic targets, and kill only poorly on TNP-modified allogeneic targets. By contrast, cells stimulated with allogeneic cells kill both allogeneic targets of the stimulating type and TNP-modified targets that are syngeneic to the responder cells. Table IX demonstrates that the specificity of these two types of T killer cell, as revealed by cold-target inhibition studies, is also different. Thus, although killing of TNP-self targets by TNP-self-activated cells is not inhibited by cold allogeneic targets, even if six independent haplotypes are mixed, killing

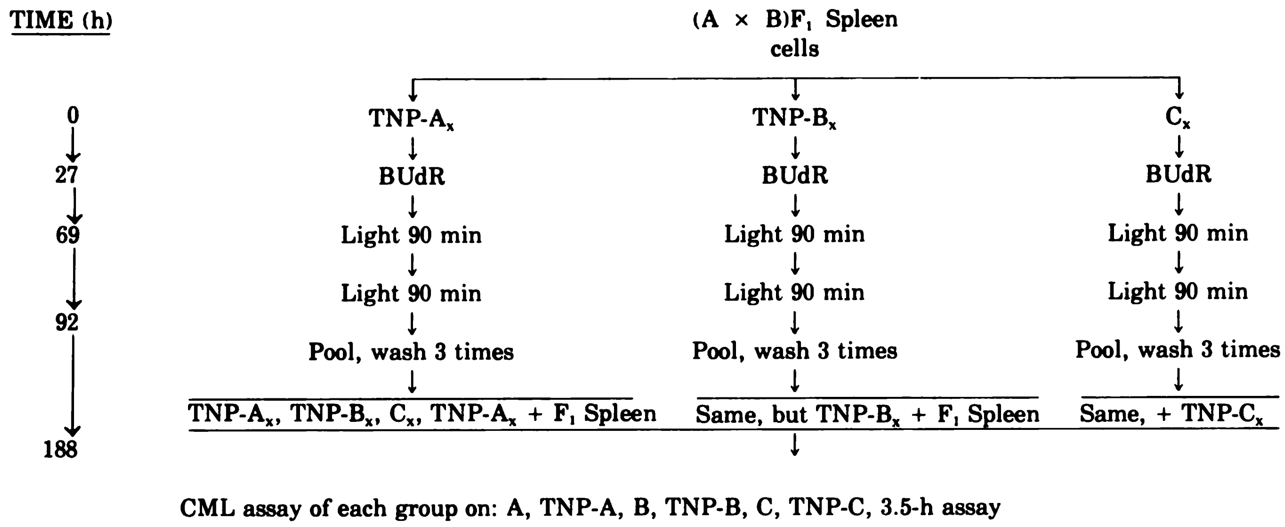
TIME (h)
0
27
69
92
188
(A × B)F$_1$ Spleen cells
TNP-A$_x$
BUdR
Light 90 min
Light 90 min
Pool, wash 3 times
TNP-A$_x$, TNP-B$_x$, C$_x$, TNP-A$_x$ + F$_1$ Spleen
TNP-B$_x$
BUdR
Light 90 min
Light 90 min
Pool, wash 3 times
Same, but TNP-B$_x$ + F$_1$ Spleen
C$_x$
BUdR
Light 90 min
Light 90 min
Pool, wash 3 times
Same, + TNP-C$_x$
CML assay of each group on: A, TNP-A, B, TNP-B, C, TNP-C, 3.5-h assay

FIG. 1. Flow diagram for BUdR and light suicide.

TABLE III

*BUdR and Light Suicide Can Selectively Eliminate Cells Giving Rise to Cytotoxicity**

Line	Stimulating cells		% specific ^{51}Cr release on targets		
	First (suiciding)	Second	TNP-CBA	DBA/2	TNP-DBA/2
1	Fresh	TNP-CBA	33.4	0.4	5.6
2	TNP-CBA	TNP-CBA	0.3	1.7	−1.7
3	TNP-A.CA	TNP-CBA	17.2	2.8	2.1
4	DBA/2	TNP-CBA	15.6	0.2	2.6
5	Fresh	DBA/2	9.0	10.7	19.4
6	DBA/2	DBA/2	8.5	3.2	7.9
7	TNP-CBA	DBA/2	10.0	16.9	18.2
8	TNP-A.CA	DBA/2	21.1	26.1	27.2
9	DBA/2	TNP-DBA/2	5.7	1.6	9.1

* Responder cells are (CBA × A.CA)F_1.

Effector to target ratio is 15:1; no significant release seen with CBA targets. "Fresh" means F_1 spleen cells prepared at the time of the "second" culture. "First" stimulator was the stimulator used to induce suicide in F_1 spleen cells. "Second" stimulator was that used in the "postsuicide" reculture of the cells. Actually, this represents a second primary culture.

TABLE IV

*F_1 T Cells Are Precommitted to Recognize TNP Associated with the H-2 of One Parent**

Line	Stimulating cells		% Specific ^{51}Cr release on targets			
	First	Second	TNP-AKR	TNP-BALB	B6	TNP-B6
10	Fresh	TNP-AKR	20.2	2.2	3.5	4.1
11	TNP-AKR	TNP-AKR	−1.6	1.2	0.9	3.0
12	B6	TNP-AKR	10.8	0.5	3.1	2.0
13	Fresh	TNP-DBA/2	1.6	8.2	0.1	2.6
14	TNP-AKR	TNP-DBA/2	1.4	7.1	2.6	7.0
15	B6	TNP-DBA/2	4.0	7.1	5.1	8.0
16	Fresh	B6	−0.8	1.7	2.2	2.4
17	TNP-AKR	B6	6.5	4.0	23.9	24.7
18	B6	B6	2.7	1.7	3.5	8.9
19	B6	TNP-B6	2.4	1.8	4.0	11.8

* Responder cells are (AKR × DBA/2)F_1.

Effector to target ratio is 40:1.

of TNP-self targets by alloreactive T cells is blocked by the appropriate, unmodified allogeneic cells.

Thus, by these criteria, at least three types of killers directed at TNP-self cells can be defined in F_1 mice: there are effector cells specific for TNP-parental cells of one or the other strain, and those stimulated by allogeneic cells. In the suicide experiments, we will present evidence that these three types of effectors come from three distinct sets of precursor cells.

Selective Suicide of Precursors Specific for Allogeneic Cells or for TNP-Parental Cells. In our experiments, we consistently find that cells responsive to TNP-parental stimulators can be suicided independently of cells responsive to allogeneic stimulators (Table III, line 2 vs. 4, 6 vs. 8; Table IV, line 10 vs. 11, 17 vs. 18; Table V, line 28 vs. 30; Table VI, line 32 vs. 35, 37 vs. 40, 42 vs. 44 and

TABLE V

*F_1 T Cells Are Precommitted to Recognize TNP Associated with the H-2 of One Parent: Failure to Detect Specific Suppressors**

Line	Stimulating cells		% Specific ^{51}Cr release on targets			
	First	Second	TNP-AKR	TNP-BALB	A.SW	TNP-A.SW
20	Fresh (A)	TNP-DBA/2	12.1	17.1	1.7	−1.2
21	TNP-DBA/2 (B)	TNP-DBA/2	1.6	0.1	−1.1	−2.3
22	(A) + (B)	TNP-DBA/2	10.2	12.6	1.2	−1.4
23	A.SW	TNP-DBA/2	1.7	−0.1	−2.3	−2.5
24	Fresh	TNP-AKR	22.0	3.2	−0.6	−1.8
25	TNP-DBA/2	TNP-AKR	13.7	−0.2	−1.5	−1.8
26	A.SW	TNP-AKR	0.3	−0.5	−2.1	−2.1
27	Fresh (C)	A.SW	25.1	14.3	36.8	31.2
28	A.SW (B)	A.SW	0.8	0.2	−1.0	−2.6
29	(C) + (B)	A.SW	7.5	4.8	21.4	8.0
30	TNP-DBA/2	A.SW	16.8	10.5	27.1	17.0

* Responder cells are (AKR × DBA/2)F_1.
Effector to target ratio is 50:1; essentially no killing on AKR or BALB targets.

TABLE VI

*Evidence of Three Distinct Precursor Cells Specific for TNP-Self**

Line	Stimulating cells		% Specific ^{51}Cr release on targets			
	First (suiciding)	Second	TNP-AKR	TNP-BALB/c	B6	TNP-B6
31	Fresh (A)	TNP-AKR	26.5	6.4	−2.2	4.1
32	TNP-AKR (B)	TNP-AKR	2.6	−0.4	−2.1	1.1
33	(A) + (B)	TNP-AKR	26.1	9.8	−0.8	2.3
34	TNP-DBA/2	TNP-AKR	16.0	0.8	−1.3	0.7
35	B6	TNP-AKR	13.1	0.3	−0.5	0.3
36	Fresh (A)	TNP-DBA/2	14.0	13.7	−0.1	2.7
37	TNP-DBA/2 (C)	TNP-DBA/2	2.6	3.7	−1.9	1.3
38	(A) + (C)	TNP-DBA/2	6.7	10.5	−1.8	2.5
39	TNP-AKR	TNP-DBA/2	5.6	1.1	−3.1	−0.2
40	B6	TNP-DBA/2	2.8	6.7	−1.8	1.4
41	Fresh (A)	B6	4.6	11.1	27.1	15.4
42	B6 (D)	B6	2.6	5.9	2.7	3.9
43	(A) + (D)	B6	3.9	4.9	18.3	12.8
44	TNP-AKR	B6	−0.5	12.0	30.8	16.1
45	TNP-DBA/2	B6	11.9	11.3	33.7	14.9
46	B6	TNP-B6	1.4	3.0	−0.5	0.8
47	Fresh B6 spleen	TNP-B6	8.2	4.3	−1.0	8.2

* Responder cells are (AKR × DBA/2)F_1.
Effector to target ratio is 37:1 except for mixture experiments where a ratio of 75:1 was used to compensate for dilution of responsive cells with unresponsive cells. Unmodified AKR and BALB/c targets showed only background lysis.

45; Table VII, lines 55 and 57 vs. 59, 61 vs. 63 and 64). Thus, we can say that our suiciding conditions are both selective and adequate for these experiments.

Loss of Response of Suicided Cells is Not Due to the Generation of Suppressor Cells. We have done several control experiments to test whether specific

TABLE VII

*BUdR and Light Suicide of F_1 T Cells Specific for TNP-Modified Parental Cells Is Selective for K and D Determinants in the Parental H-2**

Line	Stimulator cells		% Specific ^{51}Cr release on targets			
	First	Second	TNP-A.TH	TNP-A.TL	B6	TNP-B6
48	Fresh (A)	TNP-A.TH	16.2	15.4	3.4	10.4
49	TNP-A.TH (B)	TNP-A.TH	−0.9	−1.7	−0.5	0.4
50	(A) + (B)	TNP-A.TH	18.1	14.6	0.9	4.7
51	TNP-A.TL (C)	TNP-A.TH	−2.0	−0.1	0.4	0.2
52	(A) + (C)	TNP-A.TH	9.3	9.1	−2.4	0.8
53	B6	TNP-A.TH	1.1	1.3	−0.4	−0.5
54	Fresh (A)	TNP-A.TL	7.5	6.4	−0.3	1.7
55	TNP-A.TL (C)	TNP-A.TL	−2.7	−1.9	−1.7	−2.0
56	(A) + (C)	TNP-A.TL	18.5	13.6	−0.4	1.5
57	TNP-A.TH (B)	TNP-A.TL	−1.5	−1.6	−1.3	−1.0
58	(A) + (B)	TNP-A.TL	22.2	17.2	0.4	5.5
59	B6	TNP-A.TL	6.4	3.5	−0.4	0.3
60	Fresh (A)	B6	10.4	11.9	40.0	39.7
61	B6 (D)	B6	8.8	6.3	1.6	6.0
62	(A) + (D)	B6	15.7	17.0	43.0	33.0
63	TNP-A.TH	B6	16.7	12.2	31.4	32.9
64	TNP-A.TL	B6	16.5	14.4	34.4	32.7
65	B6	TNP-B6	5.3	4.9	0.9	8.8
66	Fresh B6 spleen	TNP-B6	3.2	2.6	0.4	11.9

* Responder cells are (A.TH × A.TL)F_1.

Effector to target ratio is 50:1 except for mixture groups where 100:1 was used to compensate for dilution of responsive cells 1:1 with unresponsive cells. A.TH and A.TL targets showed lysis to a low extent in a few groups, and this has been subtracted from the kill on TNP-modified targets.

TABLE VIII

Killing of TNP-Self by Alloactivated Cells

Responders	Stimulators*	% Specific ^{51}Cr release on targets			
		CBA	TNP-CBA	A.CA	TNP-A.CA
CBA	–	−3.1‡	1.9	−3.0	−2.3
CBA	TNP-CBA$_m$	−3.7	53.4	−2.5	8.6
CBA	A.CA$_m$	−3.9	15.1	32.4	38.0
A.CA	TNP-A.CA$_m$	−0.8	17.9	−5.1	32.8

* Stimulators inactivated by 30 μg/ml mitomycin C, 30 min, 37°C, followed by four washes.

‡ Mean of triplicate wells; standard errors all <2.0%.

suppressor cells could be responsible for the loss of response seen after suicide. When suicided cells are mixed 1:1 with fresh F_1 spleen cells and stimulated with the same stimulator used for the suiciding culture, to which the suicided cells can no longer respond, the response obtained is not significantly different from that expected from the fresh F_1 cells cultured alone with that stimulator (Table V, line 21 vs. 22; Table VI, line 31 vs. 33, 36 vs. 38, 41 vs. 43; Table VII, line 48 vs. 50 and 52, 54 vs. 56 and 58, 60 vs. 62). This strongly suggests that our suiciding conditions deplete responsive cells by eliminating them rather than

TABLE IX

Cold-Target Inhibition Studies of the Alloreactive Cell Killing TNP-Self Targets

Reaction being inhibited*	% ^{51}Cr Release in the presence of the inhibitors‡					
	Medium	CBA	TNP-CBA	A.CA	TNP-A.CA	Mixture§
CBA anti-TNP-CBA on TNP-CBA	53.4	42.5	14.1	–¶	37.5	38.2
CBA anti-A.CA on TNP-CBA	15.1	14.2	0	2.8	2.4	1.5
CBA anti-A.CA on TNP-A.CA	38.0	19.5	21.4	5.4	–	5.2

* Effector to target ratio is 40:1; same cells as in Table VI.

‡ Inhibitor to target ratio is 80:1.

§ Mixture of equal numbers of spleen cells from mice of haplotypes: *b, d, f, q, r, s*.

Underlined groups show significant inhibition compared with unmodified CBA blockers.

¶ Not tested.

by inducing specific suppressor cells. Nor do we have any evidence for nonspecific suppressor cells among these precultured and suicided cells (8, 17, 18), suggesting that such cells are also suicided in these cultures.

Selective Suicide of Precursors of Killer Cells Specific for TNP-Modified Cells of One or the Other Parental Strain. In most of these experiments, using F_1 responder cells derived from parental strains differing throughout the MHC, suicide with TNP-modified cells from one parental strain did not eliminate the response to TNP-modified cells of the other parental strain (Table III, line 2 vs. 3; Table IV, line 11 vs. 14; Table V, line 21 vs. 25; Table VI, line 34 vs. 37). Only one exception to this was seen (Table VI, line 32 vs. 39). However, this exception is perhaps misleading because in this experiment, cross-reactivity between TNP-AKR and TNP-DBA/2 targets was greater than expected (Table VI, line 36). The reasons for this cross-reactivity are not known, but it is interesting that this cross-reactivity at the effector level occurred only in the one experiment where cross-reactivity was also seen in the suiciding experiment.

When the K and D Loci of the Parental Strains Are Identical, Precursors for TNP-Modified Parental Cells Are Identical. We wanted to determine whether the selective suicide of F_1 responses to TNP-modified parental stimulators was due to recognition of *K*/*D* or of I region determinants. We, therefore, repeated these experiments using (A. TH × A.TL)F_1 responder cells. The two parental strains are identical at the *K* and *D* loci, but differ throughout the *I* region (Table I). In this instance, suicide with TNP-modified cells of either parental strain eliminated the response to TNP-modified cells of both parental strains (Table VII, lines 49, 51, 55, 57). Thus, selective suicide is specific for *K* and *D* locus antigens and most likely represents suicide of precursors of the cytotoxic effector cells themselves. Whether this technique is also capable of suiciding helper cells for these responses requires further experimentation.

Suicide of Cells Responsive to TNP-Parental Cells Does Not Eliminate Cells Stimulated by Allogeneic Cells to Kill TNP-Parental Targets. As noted above, allogeneic stimulators can induce cytotoxic effector cells that kill TNP-modified self targets. Such cells differ in several ways from cytotoxic effectors induced by TNP-self cells. The present experiments show that the precursors of such cells are distinct also. Thus, suicide with TNP-parental cells does not eliminate the killing of TNP-parental targets induced by allogeneic stimulators (Table III, line 5 vs. 7; Table IV, line 16 vs. 17; Table V, line 27 vs. 30; Table VI, line 41 vs. 45; Table VII, line 60 vs. 63 and 64). An exception to this is also seen in one experiment (Table VI, line 44). This latter group of cells was exceptional in other ways, as noted above, and this is the only experiment in which we have failed to see killing of TNP-parental cells after suicide when allogeneic cells are used as the second stimulators. It should be pointed out that the experiment in Table VII represents a strong argument that we are suiciding precursor cells in these experiments, and that activation of TNP-parental suicided cells with allogeneic stimulators to kill TNP-parental targets does not represent a compensation for inadequate help; in this experiment suicide was seen with either parental strain, even though they differ in the *I* region, which latter is thought to be involved in helper effects important for cytotoxic effector cell generation. Thus, we would conclude that alloreactive cells killing TNP-self targets come from different precursors than those activated by TNP-self.

Suicide with Allogeneic Cells Does Not Unmask a Response to TNP-Modified Allogeneic Cells. After suicide with allogeneic stimulators, the cells were restimulated either with allogeneic cells or TNP-modified allogeneic cells, and then tested with either allogeneic or TNP-allogeneic targets. It is essential, in such experiments, that both modified and unmodified stimulators as well as targets be used, as can be clearly seen in our results. We have very good elimination of alloreactive cells in several experiments, with no evidence of suppression. Such cells will respond to TNP-modified parental stimulators after suicide with allogeneic stimulators (see above). Nonetheless, such cells do not respond to TNP-modified allogeneic stimulators by making killer cells specific for TNP-modified allogeneic targets, when compared with their response to allogeneic unmodified stimulators (Table III, line 6 vs. 9; Table IV, line 18 vs. 19; Table VI, line 42 vs. 46; Table VII, line 61 vs. 65). In each case, the importance of the unmodified stimulator control can be seen. In the experiments in Tables VI and VII, the activity of the TNP-modified B6 stimulators was confirmed using B6 spleen cells as responders. Thus, we have no evidence that normal mice harbor significant numbers of precursor cells specific for TNP-modified allogeneic cells but normally masked by the strong response to alloantigen.

Discussion

These experiments were undertaken to define the numbers and types of precursors of cytotoxic effector cells specific for TNP-modified parental strain targets in normal (A × B) F_1 mice. Of four possible sets of precursors, we have found evidence for three. These are (*a*) precursors specific for TNP-modified cells of parental strain A; (*b*) precursors specific for TNP-modified cells of parental strain B; (*c*) precursors specific for TNP-modified parental (A or B) cells, but activated only by allogeneic stimulators and not activated by TNP-modified parental stimulators. We did not find any evidence for a fourth possible class of precursors, namely, those capable of responding specifically to TNP-modified allogeneic cells.

These data are entirely consistent with the elegant ontogenetic experiments of Zinkernagel et al. (9, 10) and of Bevan (11). These authors showed that F_1 stem cells differentiating in parental thymus preferentially respond to modified cells of the parental thymus type. They did not find evidence, even in tolerance situations, for an ability of T cells to respond to fully allogeneic-modified stimulators. This result and ours (confirmed by Schmitt-Verhulst and Shearer [19]) conflict with the results of other authors who suggested that acute depletion of alloreactive cells revealed strong reactivity toward TNP-modified allogeneic stimulators (13, 14). The reason for these differences is not clear. Wilson et al. (13) used an entirely different technique to deplete alloreactive cells, which may be responsible for the differences in the results. They also do not present data on response to unmodified allogeneic stimulators in the mouse system, but such controls were performed in parallel experiments carried out in rats and did not show lysis of TNP-modified allogeneic targets. Thomas and Shevach (14) likewise did not report controls using unmodified allogeneic cells as second stimulators; such controls were, however, performed in preliminary experiments and again did not elicit responses to TNP-modified allogeneic cells (Shevach, personal communication). Although Thomas and Shevach used essentially the same technique as we used here to eliminate alloreactive cells, the protocol and the species were different. It is clear that further experimentation is needed to clarify this crucial point.

We were surprised by the finding that alloreactive cells capable of killing TNP-modified parental cells in F_1 mice differ from F_1 cells stimulated by TNP-parental cells to kill TNP-parental targets, since we had previously speculated that such cells were identical. From this, and from the experiments of Wilson and Heber-Katz (20, 21), we had postulated that a given T cell can respond to two types of antigen: foreign MHC antigens, and modified self MHC antigens. Our results in the present experiments would suggest that this is not the case. However, it may also be that TNP-parental stimulators are relatively weak activators of cytotoxic effector cell precursors, whereas allogeneic cells are strong stimulators. If this were so, then suicide with TNP-parental cells would eliminate only a small fraction of all possible responding cells, the remainder being much more readily activatible by allogeneic cells. Experiments to test this proposition are now in progress. Recent experiments by Bevan (12) have suggested that allogeneic killers could be raised when T cells are immunized with minor alloantigens. However, these experiments only show that alloimmune cells will lyse targets bearing minor antigens which are *H-2* identical with the responder, and show cold-target inhibition data similar to that presented here; they do not directly deal with the nature of the precursors of these cytotoxic cells.

If our conclusions from these results are correct, they in turn suggest that T cells differentiate in response to self MHC antigens. Zinkernagel (9, 10) has shown that at least part of this differentiation occurs in the thymus, but there are additional steps involved that may occur postthymically as well. Whether these cells differentiate in response only to self MHC antigens, or whether their differentiation requires foreign non-MHC antigens in an inapparent form, can not be determined as yet. However, it is clear that such cells do evolve an ability to recognize self MHC antigens, whether one uses chimeric T cells or normal F_1 T cells, as in the present experiments. This commitment of self MHC precedes intentional immunization, as shown by these experiments, because if it followed immunization, TNP-modified cells from either parental strain should suicide all TNP-reactive precursors in an F_1 mouse. These results would seem to us to be more compatible with a T-cell receptor that has two sites, one specific for self MHC antigens, and one specific for the foreign, non-MHC antigen. Both types of recognition show exquisite specificity (3, 5, 22, 23). Thus, even though one cannot state conclusively from these or other experiments whether the T-cell receptor has one or two binding sites, we feel strongly that available evidence, including the results of the present experiments, favor the two-receptor site model.

Summary

The technique of antigen-driven, 5-bromo-deoxyuridine and light suicide has been adapted to eliminate the precursors of cytotoxic effector cells both for alloantigen and for 2,4,6-trinitrophenyl(TNP)-modified stimulator and target cells. Using this technique, the following observations have been made. Precursors of killer cells specific for alloantigen can be suicided independently of precursors of killer cells specific for TNP-modified self cells. The loss of activity during this procedure is not due to either specific or nonspecific suppressor cells, as judged by mixing experiments. With responder cells from F_1 animals, it has been possible to show that precursors specific for TNP-modified cells from one parent are suicided independently of precursors specific for TNP-modified cells of the other parent, but only if the parental strains differ in the *K* and *D* regions of the *H-2* complex. Cells of F_1 mice derived from *K* and *D* identical, *I* region different, parental strains were specifically suicided by TNP-modified stimulator cells from either parent. However, the cross-reactive killing of TNP-self targets induced by stimulation with allogeneic cells is not eliminated by first suiciding with TNP-parental cells, suggesting that the precursors of these two types of TNP-self killer cells are different. This is compatible with reported differences in their specificity, as confirmed in this report. Finally, deletion of alloreactive cells by this technique reveals little or no reactivity specific for TNP-modified allogeneic stimulator cells. In summary, these results strongly suggest that recognition of self MHC antigens is preprogrammed in peripheral T cells of normal animals, and is not acquired during the immunization process. They also suggest that cells specific for modified alloantigen are relatively rare in the strains of mice studied.

The authors wish to thank Ms. Deborah Bert for her technical support and Ms. Carol Sanford for her patience in typing the manuscript.

Received for publication 29 December 1977.

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ACUTE PHASE REACTANTS CERULOPLASMIN AND HAPTOGLOBIN AND THEIR RELATIONSHIP TO PLASMA PROSTAGLANDINS IN RABBITS BEARING THE VX$_2$ CARCINOMA*

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Results of a series of studies on the cause of the hypercalcemia that occurs in mice bearing the HSDM$_1$ fibrosarcoma (1-6) and in rabbits carrying the VX$_2$ carcinoma (7-9) have led us to conclude that these two tumors synthesize and secrete large amounts of prostaglandin E$_2$ (PGE$_2$) into plasma.[1] PGE$_2$ is a potent bone resorption-stimulating agent in vitro (2, 10), and this prostaglandin and its metabolites are found in elevated concentrations in the plasma of tumor-bearing animals (2, 3, 6-8, 11). Because of the rapid clearance and metabolism of PGE$_2$, measurements in plasma of the metabolite, 13,14-dihydro-15-keto-PGE$_2$(PGE$_2$-M), give a more accurate estimate of PGE$_2$ secretion than do measurements of the primary prostaglandin itself (6, 8, 11, 12). Studies on the time-course of the development of elevated plasma calcium concentrations and hyperprostaglandinemia, as well as investigations using two inhibitors of prostaglandin synthesis, indomethacin (1-3, 7) and hydrocortisone (6, 8, 13), support the hypothesis that the hypercalcemic syndrome in these tumor-bearing animals is due to the secretion of PGE$_2$ by the tumor. A similar pathophysiologic mechanism may explain in part the hypercalcemia that occurs in certain patients with cancer (14-16).

The present investigation was initiated because of the observation that the plasma from rabbits bearing the VX$_2$ carcinoma became faintly blue about 1 wk after tumor implantation, and this color increased markedly and became intense by 3-4 wk. The time-course of the appearance and increase in the blue color in plasma was similar to that which we had previously noted for PGE$_2$-M. We therefore undertook to identify the blue material in plasma and to examine the relationship of its increase to prostaglandin metabolism. Our findings indicate that the material is ceruloplasmin, that its rise correlates closely with plasma concentrations of PGE$_2$-M, and that both PGE$_2$-M and ceruloplasmin increase in the plasma of tumor-bearing rabbits before the development of hypercalcemia.

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* Supported in part by research grants AM 10206, CA 17309, CA 19416, and AI 14157 from the National Institutes of Health.

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[1] *Abbreviations used in this paper:* dl, deciliter; PGE$_2$, prostaglandin E$_2$; PGE$_2$-M, 13,14-dihydro-15-keto-PGE$_2$.

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Ceruloplasmin is a blue, copper-containing α_2-globulin which possesses intrinsic oxidase activity (17–19). Its concentration in plasma is known to vary in a variety of physiologic states and in disease (18). Decreased plasma levels have been detected in Wilson's disease, nephrosis, and malabsorption syndromes, and elevated concentrations are seen in acute and chronic infections, rheumatoid arthritis, pregnancy, during estrogen administration, and in patients with a variety of different tumors. The mediators which control ceruloplasmin synthesis and secretion by the liver in health and disease have not been clearly defined.

Plasma concentrations of haptoglobin, another α_2-globulin which binds hemoglobin, are known to vary in parallel with ceruloplasmin in certain states of disease, notably acute and chronic infection, and in patients with certain malignancies (20). Furthermore, it has been reported that administration of PGE_1 can elevate the concentration of haptoglobin in the serum of rabbits (21). We therefore measured haptoglobin as well as ceruloplasmin in the plasma of rabbits bearing the VX_2 carcinoma, and we found elevations which paralleled those of the copper-containing protein. A preliminary presentation of these findings has been made (22).

Materials and Methods

Animals. The VX_2 carcinoma was passed serially in female albino rabbits by methods previously described in detail (7). In experiments in which rabbits were treated with indomethacin from the time of tumor implantation, the drug was administered orally in an average daily dosage of 10–40 mg/rabbit/24 h. Indomethacin was incorporated into a known amount of pulverized Purina Lab Chow for Rabbits (Ralston Purina Co., St. Louis, Mo.) which the animals consumed essentially completely each 24 h (7). In experiments in which rabbits were treated with indomethacin intermittently after the development of hypercalcemia, the drug was suspended in 15% gelatin and administered by subcutaneous injection twice a day in a daily dosage of 10–20 mg/rabbit. The animals weighed 2.5–3.0 kg. For periods up to 4 wk after tumor implantation, these rabbits did not become azotemic as determined by measurements of plasma urea nitrogen and creatinine. The exact schedules by which indomethacin was given are indicated in Results.

Blood Collection. Blood was collected from a marginal ear vein or by cardiac puncture into heparinized tubes or syringes. Plasma was separated immediately by centrifugation at 4°C.

Ceruloplasmin. The concentrations of ceruloplasmin in plasma were estimated by two independent methods. The first method used the procedure of Sunderman and Nomoto (23) which depends on the *p*-phenylenediamine oxidase activity of ceruloplasmin. The standard was human ceruloplasmin, type III, from Sigma Chemical Co., St. Louis, Mo. (lot 114C-0237-1). The second method used Laurell's electroimmunoassay technique (24, 25). The antiserum was prepared against purified human serum ceruloplasmin in goats, and it was obtained from Atlantic Antibodies, Westbrook, Maine. This antiserum cross-reacted sufficiently with rabbit ceruloplasmin to provide easily identified rockets. The immunoassay results were expressed as a percentage of the basal ceruloplasmin concentration before tumor implantation for each animal.

Immunochemical Measurements of Plasma Haptoglobin and Albumin. The concentrations of haptoglobin and albumin in plasma were measured by electroimmunoassay (24, 25) using antisera prepared against analogous purified human serum proteins in goats (Atlantic Antibodies). As with ceruloplasmin, these antisera cross-reacted sufficiently with the corresponding rabbit plasma proteins to give clear rockets. The results were expressed as a percentage of the basal concentration of that protein before tumor implantation for each animal.

Prostaglandin Metabolite. The metabolite of PGE_2, PGE_2-M, was measured in plasma by radioimmunoassay (26). The anti-PGE_2-M cross-reacted with 13,14-dihydro-PGE_2, 15-keto-PGE_2, 13,14-dihydro-15-keto-$PGF_{2\alpha}$, 13,14-dihydro-15-keto-PGA_2, PGE_2, and PGA_2 0.2, 7.0, 5.0, 0.4, 0.1, and 0.08%, respectively (26). Plasma for PGE_2-M assay was extracted with 3 vol of methylal-ethanol and concentrated as described previously for human samples (27). Several extracts of rabbit plasma were assayed with anti-13,14-dihydro-15-keto-$PGF_{2\alpha}$. The 13,14-dihydro-15-keto-PGE_2 gave a cross-reaction with this anti-$PGF_{2\alpha}$ metabolite of ≅3% (28). These simultaneous radioimmunoassays demonstrated that it was the PGE_2 metabolite, not the $PGF_{2\alpha}$ metabolite, that was being measured in the experiments in this report. Similar quantitative results for plasma PGE_2-M have been measured in rabbits basally and during the first 3 wk after VX_2 tumor implantation using high performance liquid chromatography and gas chromatography-mass spectrometry (11). The sensitivity of the radioimmunoassay method was 15 pg of PGE_2-M/ml rabbit plasma, and the precision of a measured value was ±20%.

Calcium. The concentration of calcium in plasma was measured in duplicate by automatic fluorometric titration with a Corning model 940 calcium analyzer (Corning Medical, Corning Glass Works, Medfield, Mass.).

Statistical Method. Where appropriate, when groups of rabbits were studied, the results were subjected to an analysis of variance, and the standard errors were calculated from the residual error term of that analysis.

Results

Plasma Ceruloplasmin. The concentration of ceruloplasmin in the plasma of 12 normal control rabbits was 37 ± 4 mg/deciliter (dl) (mean ± SE), as measured by its *p*-phenylenediamine oxidase activity. This value is similar to that found in normal human serum, 31.5 ± 5 mg/dl (mean ± SD), using the same technique (23).

The rise in plasma ceruloplasmin as a function of time after implantation of VX_2 tumor cells in three rabbits is shown in Fig. 1. A marked increase of 10 to 20 times the basal concentration was seen 3–4 wk after tumor implantation. There was generally good agreement between the results obtained on the same samples by both the chemical and immunological assay methods (Fig. 1). The largest discrepancy observed in over 25 rabbits studied was that seen in rabbit 224 (Fig. 1 C) between 1.5 and 2.5 wk.

Plasma Haptoglobin and Albumin. Fig. 2 A shows that plasma haptoglobin rose markedly after tumor implantation in three rabbits. In the same animals, there was little or no change in the concentration of albumin in plasma (Fig. 2 B). Similar results were observed in five other rabbits.

Relationship of Plasma Ceruloplasmin to Plasma Prostaglandin Metabolites and Calcium. We have reported previously that PGE_2-M rises rapidly in the plasma of rabbits bearing the VX_2 carcinoma (8). The rise in PGE_2-M is much greater than the increase in PGE_2 itself (8, 11) and it precedes the elevation of plasma calcium concentration. The time-courses of changes in the concentrations of PGE_2-M, ceruloplasmin, and calcium in plasma in three rabbits bearing VX_2 carcinomas are shown in Fig. 3. In each rabbit, plasma PGE_2-M and ceruloplasmin rose earlier after tumor cell implantation than did plasma calcium. Plasma PGE_2-M and ceruloplasmin rose between wk 1 and 2, whereas plasma calcium did not rise above basal concentrations until 2–3 wk after tumor implantation (Fig. 3). These relationships are more clearly displayed when the results obtained in a group of rabbits are pooled and plotted together (Fig. 4). It is seen that the rises in plasma PGE_2-M and ceruloplasmin occur at approximately the same time, and that both precede the increase in plasma calcium. At 1 wk, plasma PGE_2-M was 340 ± 100 pg/ml (mean ± SE) as compared to a control value of 100 ± 30 pg/ml ($P < 0.05$), and plasma ceruloplasmin was 55 ± 5 mg/dl as compared to a control value of 37 ± 4 mg/dl ($P < 0.05$).

Effects of Indomethacin on Plasma Ceruloplasmin and Haptoglobin. The

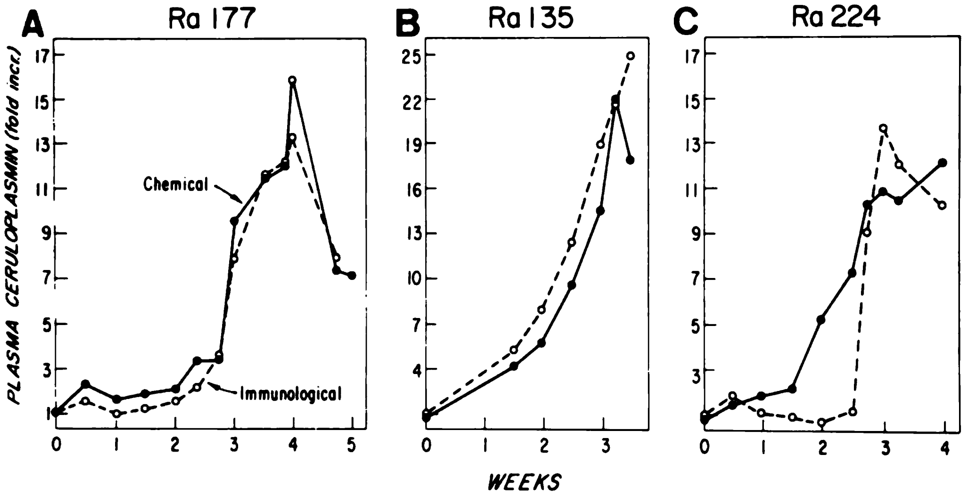

FIG. 1. Time-course of rise of plasma ceruloplasmin in three rabbits: (A), Ra 177; (B), Ra 135; and (C), Ra 224 after implantation of VX_2 tumor cells at 0 wk. The same plasma samples were assayed by the chemical (●——●) and immunological (O- - -O) assay procedures describod in Materials and Methods. The samples for immunoassay were coded and ceruloplasmin was measured without knowledge of the experimental protocol. The data are plotted as fold increase (incr.) above the control basal value (set at 1.0) for each rabbit measured before tumor implantation.

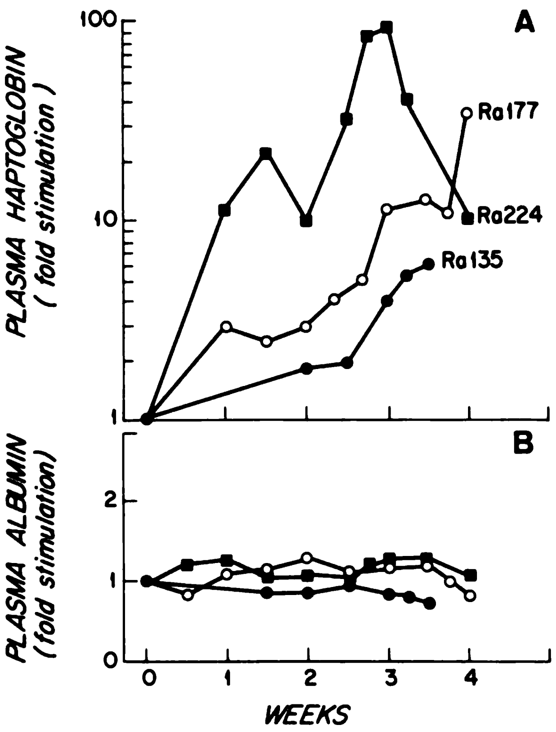

FIG. 2. Time-course of change in plasma haptoglobin (A) and plasma albumin (B) in three rabbits: Ra 135 (●), Ra 224 (■), and Ra 177 (O) after implantation of VX_2 tumor cells at 0 wk. The data are plotted as fold stimulation above the control basal value (set at 1.0) for each rabbit measured before tumor implantation.

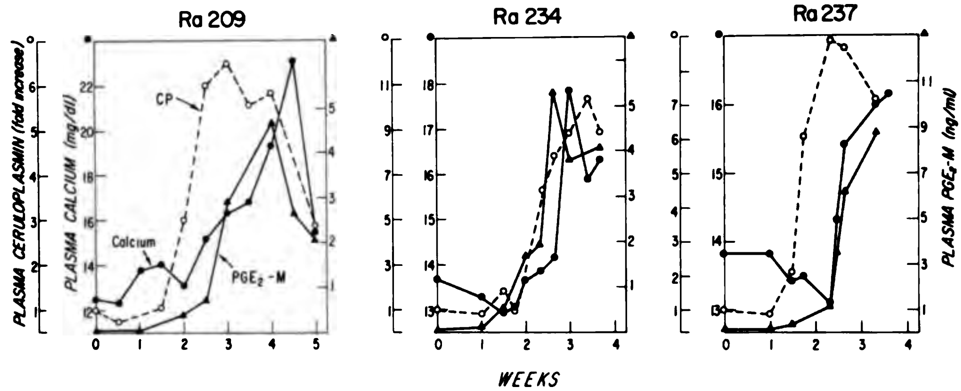

FIG. 3. Time-courses of rises of plasma PGE_2-M (▲——▲), ceruloplasmin (O- - -O), and calcium (●——●) in three rabbits (Ra 209, Ra 234, and Ra 237) after implantation of VX_2 tumor cells at 0 wk. The data for PGE_2-M and calcium are given as absolute concentrations in plasma, while those for ceruloplasmin (CP) are plotted as fold increase above the control basal value (set at 1.0) for each rabbit measured before tumor implantation.

anti-inflammatory drug indomethacin is a potent inhibitor of prostaglandin synthesis (29), and when administered to rabbits bearing the VX_2 carcinoma, it prevents the rise in plasma calcium (7), plasma PGE_2 (7), and plasma PGE_2-M (8), as well as decreasing the PGE_2 content of the tumor (7). Indomethacin also inhibits the synthesis of PGE_2 by strains of VX_2 tumor cells in culture (7).

In two rabbits, indomethacin administration was begun at the time of tumor cell implantation, and plasma calcium, ceruloplasmin, and haptoglobin concentrations were measured (Fig. 5). In contrast to the expected large rises in all three plasma components (Figs. 1–4), there was no increase in plasma calcium, and little or no change in plasma ceruloplasmin and haptoglobin (Fig. 5). If tumor-bearing rabbits were permitted to develop elevated concentrations of PGE_2-M and ceruloplasmin in plasma and were then treated with indomethacin, the continued rise in both plasma components was inhibited, or both components fell in parallel (Fig. 6). In Fig. 6 A, temporary cessation of indomethacin administration was followed by an increase in plasma PGE_2-M and ceruloplasmin, both of which were decreased by a second course of treatment with indomethacin.

Discussion

From the results presented in this communication we conclude that the concentrations of the acute phase reactants, ceruloplasmin and haptoglobin, are elevated in the plasma of rabbits bearing the VX_2 carcinoma. The validity of this conclusion depends on the specificity of the findings and of the assay methods used. The clue that initiated our studies was the blue color of the plasma of tumor-bearing rabbits. Ceruloplasmin is a blue protein (17–19). The color of the rabbit plasma was not characteristic of bilirubin, the rabbits had neither hepatic metastases nor bile duct occlusion, and plasma bilirubin was not elevated (unpublished data). All plasma proteins were not elevated nonspecifically because plasma albumin remained unchanged (Fig. 2). Ceruloplasmin was measured in plasma by two independent techniques, a chemical method

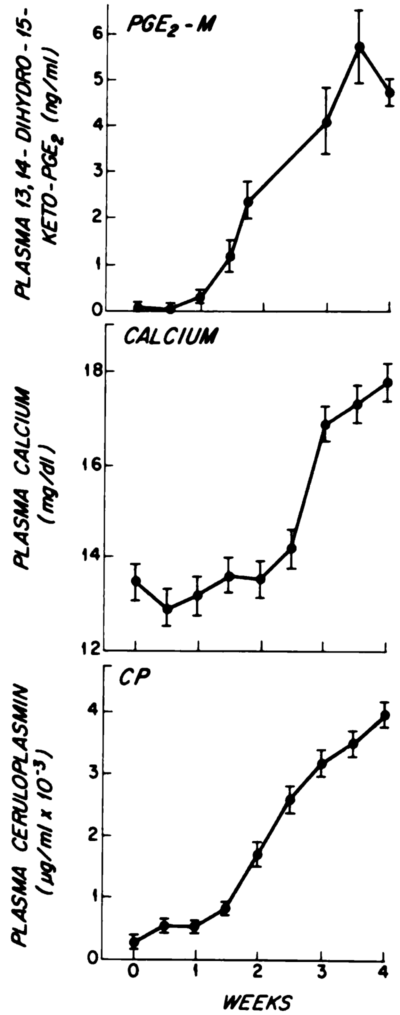

FIG. 4. Time-courses of changes in plasma PGE$_2$-M, calcium, and ceruloplasmin (CP) in a group of rabbits after implantation of VX$_2$ tumor cells at 0 wk. Each point gives the mean value of 7-12 rabbits and the bars give the SE. Rises above control values for PGE$_2$-M and ceruloplasmin were statistically significant ($P < 0.05$) at 1 wk and highly significant ($P < 0.001$) at 1.5 wk, whereas the rise in plasma calcium did not become statistically significant until 3 wk.

utilizing the *p*-phenylenediamine oxidase activity of ceruloplasmin, and an immunological assay, and the results of the two techniques were in good agreement. Furthermore, the subjective assessment of the increase in intensity of blue color in plasma correlated well with the results of both quantitative assay methods.

The time-courses of the rises in plasma ceruloplasmin and PGE$_2$-M in rabbits bearing the VX$_2$ carcinoma were very similar, and both clearly preceded the increase in plasma calcium (Fig. 4). We suggest the following hypothesis to explain our findings. The VX$_2$ tumor synthesizes and secretes large amounts of

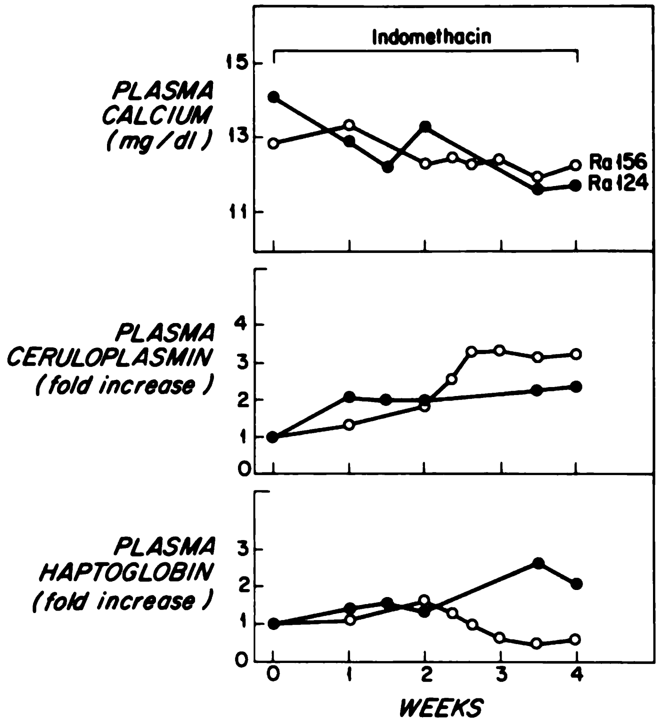

FIG. 5. Two rabbits, Ra 124 (●) and Ra 156 (○), were implanted with VX_2 tumor cells at 0 wk, and indomethacin (40 mg/rabbit/day, orally) therapy was begun immediately. Plasma calcium, ceruloplasmin, and haptoglobin were measured at intervals as described in Materials and Methods and plotted as described in the legends to previous figures.

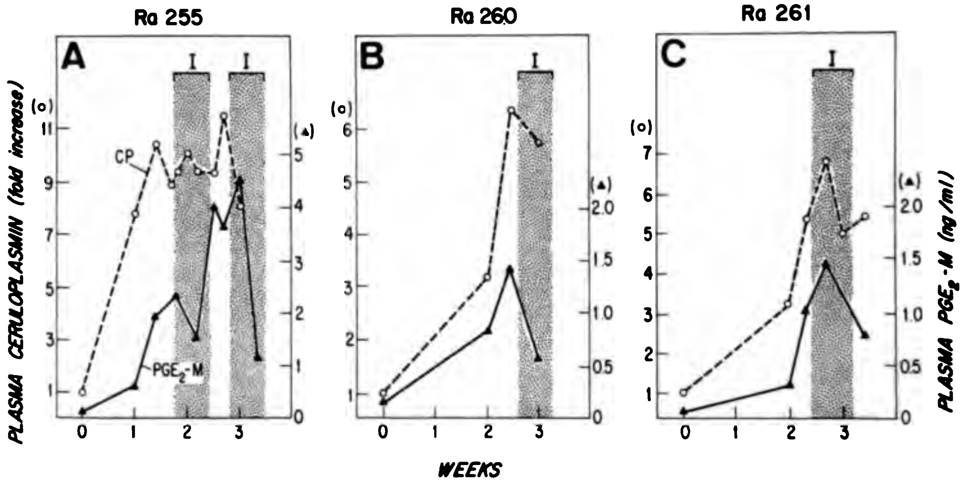

FIG. 6. Time-courses of rises of plasma PGE_2M (▲——▲) and ceruloplasmin (CP) (○- - -○) in three rabbits: (A), Ra 255; (B), Ra 260; and (C), Ra 261, after implantation of VX_2 tumor cells at 0 wk. The rabbits were treated intermittently with indomethacin (10–20 mg/rabbit/day). Indomethacin administration is indicated at the top of each panel by I over the shaded area. The data for plasma ceruloplasmin are plotted as fold increase above the control basal value (set at 1.0) for each rabbit measured before tumor implantation.

PGE_2 (7, 8, 11). Of the products of arachidonic acid metabolism secreted into plasma, PGE_2 is best measured as the accumulated metabolite, PGE_2-M because of the rapid clearance and metabolism of PGE_2 itself (8, 11, 12). One or more of these arachidonic acid metabolites, possibly PGE_2 or PGE_2-M, acts on the liver to stimulate the synthesis and secretion of ceruloplasmin (and also haptoglobin). This effect is more rapid and/or more sensitive to circulating arachidonate metabolites than is the action of PGE_2 on bone; thus the increase in plasma ceruloplasmin occurs before the hypercalcemia. We acknowledge that we have at this time no experimental evidence that the effect of the arachidonate metabolite is a direct action on the liver; it could be occurring indirectly via some additional mediator. Nevertheless, such a metabolite would appear to be a relevant intermediate in the pathway between tumor and hyperceruloplasminemia because its synthesis was inhibited by indomethacin, and there was little or no rise in plasma ceruloplasmin (or haptoglobin) in the presence of indomethacin. The validity of this interpretation depends on the assumption that the doses of indomethacin used did not have effects on ceruloplasmin (or haptoglobin) synthesis, release, or metabolism that are independent of the actions of the drug on prostaglandin synthesis. To our knowledge, no such effects of indomethacin have been reported, and no changes in plasma albumin concentrations were noted by us in rabbits treated with indomethacin.

The biological significance of our findings is of possible general interest. A body of evidence has accumulated that supports the view that a number of aspects of the inflammatory response are mediated via arachidonic acid metabolites and that the anti-inflammatory actions of aspirin-like drugs are due to their inhibitory effects on the fatty acid cyclooxygenase (30–32). In this context, the frequent association of elevated plasma concentrations of ceruloplasmin and haptoglobin with acute and chronic inflammatory processes is noteworthy. We have no evidence to suggest that in generalized inflammatory diseases the plasma concentrations of PGE_2, PGE_2 metabolites, or other metabolites of arachidonic acid are elevated, although the tissue levels may be high at localized sites of inflammation. On the other hand, inflammatory stimuli appear to enhance the synthesis and release of PGE_2, and possibly other metabolites of arachidonic acid, from macrophages (33). Thus it is possible that certain inflammatory stimuli lead to elevations in plasma of acute phase reactants, including ceruloplasmin and haptoglobin, via a pathway which depends on arachidonic acid metabolism. Consistent with this hypothesis is the observation that systemically administered PGE_1 causes a marked rise in serum haptoglobin in the rabbit (21).

In the case of tumors associated with elevations of plasma ceruloplasmin and haptoglobin, our findings indicate that, at least in the specific instance of the VX_2 carcinoma, the rise in these two acute phase reactants occurs in animals bearing a PGE_2-producing tumor. The large magnitude (10- to 20-fold) and early rise in plasma ceruloplasmin in rabbits carrying the VX_2 carcinoma indicate that this easily measured plasma protein may be used to monitor tumor presence and possibly the effects of anti-tumor therapy. Whether or not these observations could be extended to certain human tumors remains uncertain because of the probable multiplicity of factors controlling acute phase reactants in human subjects.

Summary

Results of previous studies have shown that the VX_2 carcinoma in rabbits synthesizes large amounts of prostaglandin E_2 (PGE_2). PGE_2 secreted by the tumor is rapidly metabolized and can be measured in plasma as the metabolite 13,14-dihydro-15-keto-PGE_2 (PGE_2-M). We have previously proposed that the hypercalcemia that occurs in rabbits bearing the VX_2 carcinoma is due to excessive secretion of PGE_2 by the tumor and its subsequent action on the skeleton as a bone resorption-stimulating factor. In the course of these studies, we noted that the plasma of rabbits bearing the VX_2 carcinoma became blue about 1 wk after tumor implantation. The intensity of the color increased markedly thereafter. We therefore measured ceruloplasmin in plasma by both chemical and immunological assay methods. Plasma ceruloplasmin and PGE_2-M rose in parallel (within 7–10 days) and preceded by 7–10 days the development of hypercalcemia. 2 wk after tumor implantation, plasma PGE_2-M and ceruloplasmin had risen about 20- and 6-fold, respectively, while the rise in plasma calcium was just beginning. Indomethacin, an inhibitor of prostaglandin synthesis, given from the time of tumor implantation prevented completely the hypercalcemia and largely inhibited the rise in ceruloplasmin. When given after hyperprostaglandinemia had developed, indomethacin produced a fall in both PGE_2-M and ceruloplasmin. A rise in plasma haptoglobin concentrations similar to that seen for ceruloplasmin was also observed. No changes in plasma albumin concentrations occurred. We conclude that the acute phase reactants ceruloplasmin and haptoglobin rise rapidly in the plasma of rabbits bearing the VX_2 carcinoma, and that this increase is related to arachidonic acid metabolism in these animals. It is possible that arachidonic acid metabolites also play a role in the elevations of these two plasma proteins observed in certain patients with malignant tumors.

We thank Yolanda Santo and Diane Balavitch Banda for their expert assistance, and Elizabeth A. Moore for her statistical calculations.

Received for publication 5 December 1977.

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$G_{(RADA1)}$: A NEW CELL SURFACE ANTIGEN OF MOUSE LEUKEMIA DEFINED BY NATURALLY OCCURRING ANTIBODY AND ITS RELATIONSHIP TO MURINE LEUKEMIA VIRUS*

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Several systems of cell surface antigens have now been recognized serologically on leukemia cells of the mouse (1). The technique that has played the major role in the detection and analysis of these antigenic systems is the cytotoxic test, originally developed by Gorer and O'Gorman (2), in which target cells are lysed by specific antibody in the presence of a suitable source of complement. Because antigens specified by the *H*-2 complex are present on virtually all mouse cells, discovery of other systems of cell surface antigens depended on immune sera that were produced in H-2 compatible combinations or were rendered free of H-2 antibody by in vitro or in vivo absorption, or on serological test procedures that eliminated the contribution of H-2 antibody. In this way, three general categories of cell surface antigens, in addition to H-2, have been serologically defined on leukemia cells (1).

(*a*) Differentiation alloantigens, e.g., Thy-1 and the Lyt series that signify the origin of the leukemia from precursors undergoing T-cell differentiation in the thymic environment.

(*b*) Antigens of the TL[1] (thymus-leukemia) series that occur either as differentiation alloantigens (TL^+ leukemias occurring in TL^+ strains of mice) or as a consequence of the derepression or activation of silent TL genes (TL^+ leukemias occurring in TL^- strains of mice).

(*c*) Murine leukemia virus (MuLV)-related antigens that owe their origin to the genome of endogenous viruses belonging to the MuLV family. The two antigens in this category that have been best defined serologically are the G(Gross) cell surface antigen (GCSA) and the G_{IX} antigenic system, both of which have now been related to structural components of MuLV.

With the recognition that MuLV exists in a highly polymorphic state in the mouse, it might be expected that this would be reflected in the existence of an extensive range of antigenically distinct MuLV-related cell surface antigens. In this report we define by means of a naturally occurring antibody present in

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* Supported by National Institutes of Health grants CA-08748 and CA-16599.

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[1] *Abbreviations used in this paper*: C, complement; FMR, Friend, Moloney, and Rauscher MuLV; GCSA, Gross cell surface antigen; MCF, mink cell focus-inducing MuLV; MuLV, murine leukemia virus; TL, thymus-leukemia antigen.

normal mouse serum a new MuLV-related specificity, designated $G_{(\mathrm{RADA1})}$, with properties that clearly distinguish it from GCSA and G_{IX}.

Materials and Methods

Mice. Random bred Swiss Ha/ICR mice were obtained from Millerton Farms (Millerton, N. Y.) or Charles River Breeding Laboratories (Wilmington, Mass.). NZB/BlNJ (=NZB) and RF/J mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). All other mice came from our colonies.

Antisera. Preparation and reactivity of the polyvalent anti-MuLV serum ([W/Fu × BN]F_1 rat anti-MuLV-induced W/Fu rat leukemia [C58NT]D [=anti-NTD]) and the GCSA typing serum (C57BL/6[=C57BL] anti-AKR spontaneous leukemia K36) have been detailed elsewhere (3–5).

Antisera against MuLV structural proteins p15, p30, and gp70 were made according to the method of Fleissner et al. (6). Their specific reactivity was shown by radioimmunoprecipitation tests with ^{3}H-amino acid-labeled MuLV proteins.

Cells. The A strain leukemia, RADA1, was induced by X-irradiation in 1962 and has been passaged in the ascites form in the strain of origin (7). A description of the other transplantable tumor lines has been given in previous publications from our laboratory (5, 7–9).

Complement (C)-Dependent Cytotoxicity Test. Equal volumes (50 μl) of target cells (5 × 10[illegible]/ ml), antiserum (serial dilutions), and appropriately diluted rabbit serum (C source) were mixed and incubated for 45 min at 37°C. Viability counts were made in the presence of trypan blue. The diluent in all tests was medium 199 (Grand Island Biological Co., Grand Island, N.Y.). Preselected rabbit serum diluted 1:8 was used in all tests with leukemia cells and at a 1:15 dilution with normal thymocytes.

Qualitative Absorption Test. Antiserum (dilution determined by preliminary cytotoxic tests) and washed, packed cells were mixed at a ratio of 2:1 and incubated for 30 min at 4°C. Generally, the dilution of antiserum used in absorption tests was two serial dilutions below its end point (50% cells lysed). After removing the absorbing cells by centrifugation at 900 *g*, the supernate was tested for residual cytotoxic activity on the appropriate target cells (5, 10).

Quantitative Absorption Test. After absorbing 50 μl of diluted antiserum with a range of counted numbers of cells for 30 min at 4°C, the residual cytotoxic activity of the absorbed serum for RADA1 cells was determined (4, 10).

Induction of MuLV-Related Cell Surface Antigens by MuLV In Vitro. $G_{(\mathrm{RADA1})}$, G_{IX}, and GCSA induction was assayed by the ability of MuLV-infected tissue culture cells to absorb cytotoxic activity from reference antisera (10). The cells used for absorption were confluent cultures of uninfected control cells or productively infected cells. Cultures were washed once with Ca^{++} and Mg^{++} free phosphate-buffered saline and dispersed by incubation with 0.05% EDTA for 5 min at room temperature. Cells were scraped from the surface of 100-mm Petri dishes and harvested by centrifugation at 500 *g* and washed two times in Eagle's Minimal Essential Medium. After a final wash in medium 199, the cells were packed by slowly increasing the speed of centrifugation to 900 *g* over a period of 10 min.

Ecotropic viruses were propagated in mouse SC-1 cells; xenotropic and mink cell focus-inducing (MCF) viruses were propagated in mink (CCL64) cells. The histories of the viruses studied are given in reference 10 and in the footnotes to Table III.

Inhibition Tests with MuLV Proteins. Individual MuLV proteins p10, p12, p15, p30, and gp70 were purified from MuLV(Gross) by the method of Fleissner (12) and resuspended in Tris-hydrochloride pH 7.6 at a concentration of 200 μg/ml (Fig. 6 A) and 1,500 μg/ml (Fig. 6 B). Equal volumes (20 μl) of viral protein (serial dilutions) and 1:12.5 diluted antiserum (final dilution 1:25) were mixed and incubated for 30 min on ice. 50 μl of a suspension of target cells (5 × 10^6/ml) was then added, and incubation continued for 30 min on ice. The cells were washed once in 1.5 ml of medium 199, resuspended in 100 μl of diluted C, and incubated for 30 min at 37°C, followed by viability counts in the presence of trypan blue (11).

Immunoprecipitation of Labeled Membrane Proteins from Cell Lysates and Lysed Virions. The method of lactoperoxidase-catalyzed radioiodination of viable cell surface proteins and MuLV proteins has been described in detail elsewhere (11). After lysis of cells with Nonidet P-40 (Shell Chemical Co., New York), immunoprecipitation of reactive labeled surface components from a lysate of 10^7 cells was accomplished with 20 μl of undiluted mouse antiserum and 500 μl of goat

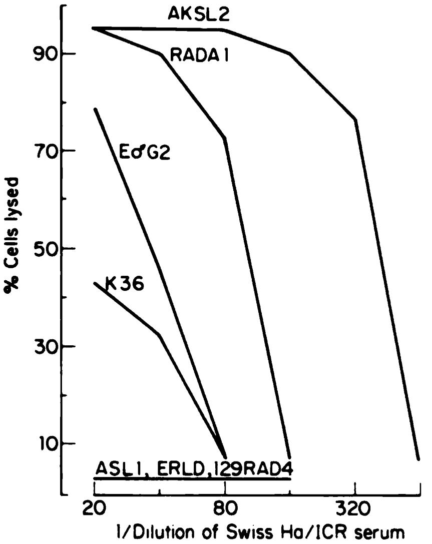

FIG. 1. Cytotoxic tests with a panel of seven transplanted mouse leukemias. Demonstration of naturally occurring cytotoxic antibody in Swiss Ha/ICR mouse serum.

anti-mouse Ig serum. Labeled polypeptides in the immunoprecipitates were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and 2-mercaptoethanol in cylindrical gels (10 cm length, 7.5% acrylamide) for 3 h at 3 mA/gel, according to the method of Laemmli (13).

Results

Serological Definition of the $G_{(RADA1)}$ Antigen. The $G_{(RADA1)}$ system was detected during a survey of normal mouse sera for naturally occurring cytotoxic antibody to leukemia cells. A proportion of random bred Swiss Ha/ICR mice were found to have such antibody, and sera from mice with the highest titer were individually tested on a panel of transplanted leukemia lines. Fig. 1 illustrates a test with seven leukemias and shows that the Swiss serum has strong cytotoxic reactivity with AKSL2, a spontaneous leukemia of AKR origin, and RADA1, an A strain leukemia originally induced by X-ray. Reactivity was intermediate with E♂G2, a leukemia induced by passage A Gross virus in C57BL mice, and K36, a spontaneous leukemia of AKR origin. No cytotoxicity was observed with ASL1, an A strain spontaneous leukemia or ERLD and 129RAD4, two X-ray-induced leukemias of C57BL or 129 origin, respectively. Absorption tests with RADA1 as the target cell were consistent with the results of direct cytotoxic tests (Fig. 2); RADA1, E♂G2, AKSL2, and K36 absorbed reactivity, whereas ERLD, ASL1, and 129RAD4 failed to do so. This result indicated that E♂G2, AKSL2, and K36, although differing in their sensitivity to the Swiss cytotoxic antibody, nevertheless shared the same antigen or spectrum of antigens with RADA1. Further absorption tests with AKSL2 or E♂G2 rather than RADA1 as the target cells confirmed this conclusion and suggested the detection of a single antigenic system. Because the surface phenotype of this leukemia panel is well defined, it was also possible to conclude that the antigen detected by the Swiss serum was serologically unrelated to any

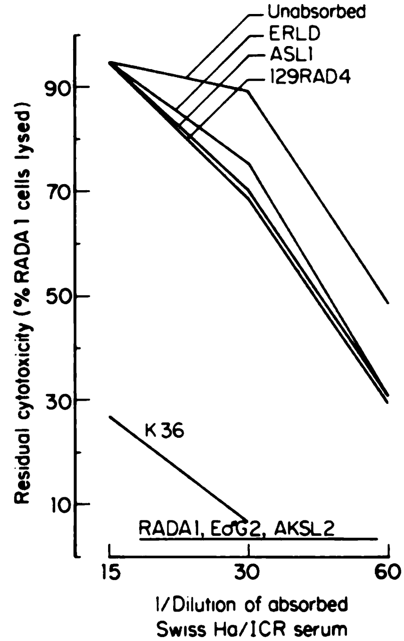

FIG. 2. Qualitative absorption tests to determine the specificity of naturally occurring cytotoxic antibody for RADA1 target cells in Swiss Ha/ICR serum. The absorption capacity of these seven leukemias parallels their sensitivity to the Swiss natural antibody in direct cytotoxic tests (see Fig. 1).

previously defined cell surface alloantigen or MuLV-related antigen. Participation of known alloantigens is excluded by the fact that ASL1, which shares all known A strain alloantigenic systems with RADA1 (H-2, TL, Lyt series, Thy-1), is not lysed by the Swiss serum, nor can it remove cytotoxic reactivity for RADA1 in absorption tests. The new specificity is also unrelated to the two MuLV-related cell surface antigens, GCSA and G_{IX}, because RADA1 lacks the GCSA determinant, and 129RAD4, which is G_{IX}^{+}, lacks the determinant detected by the Swiss serum.

RADA1 was chosen as the prototype target cell in subsequent analysis of the system. As the RADA1 antigen was found to bear a close relation to MuLV (see below), it was named $G_{(RADA1)}$ in conformity with our precedent of designating MuLV-related cell surface antigens G for Ludwik Gross who discovered this class of viruses.

Presence of $G_{(RADA1)}$ in Normal and Preleukemic Tissues

Absorption tests indicated that normal thymocytes of 2-mo-old AKR mice expressed $G_{(RADA1)}$, even though these cells were not lysed by anti-$G_{(RADA1)}$ sera in direct cytotoxic tests. For this reason, the distribution of $G_{(RADA1)}$ in normal tissues was investigated by absorption procedures.

TISSUE DISTRIBUTION OF $G_{(RADA1)}$ IN NORMAL AKR MICE. Qualitative absorption tests detected $G_{(RADA1)}$ in the thymus, spleen, bone marrow, lymph nodes, and liver of 2-mo-old AKR mice. Brain, red blood cells, and kidney typed $G_{(RADA1)}^{-}$. Quantitative absorption analysis showed that spleen cells expressed higher levels of $G_{(RADA1)}$ than other lymphoid or hematopoietic AKR cells; bone

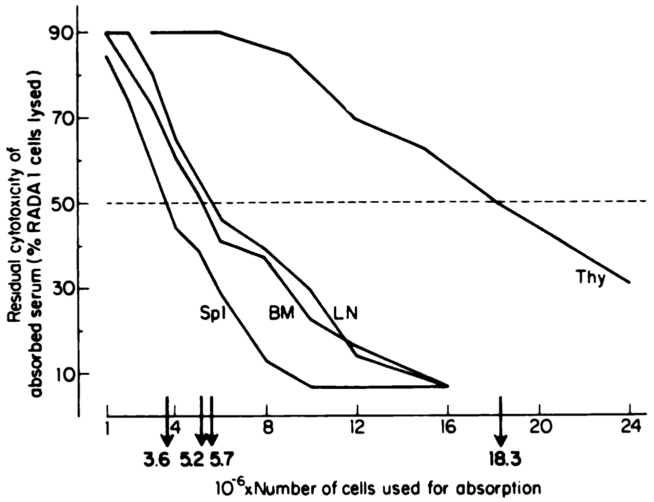

FIG. 3. Quantitative absorption tests for $G_{(\mathrm{RADA1})}$ expression on cells from spleen (Spl), bone marrow (BM), lymph nodes (LN), and thymus (Thy) of 2-mo-old AKR female mice. Arrow (↓) indicates numbers of cells that reduce the cytotoxic activity of the diluted $G_{(\mathrm{RADA1})}$ typing serum to 50% RADA1 lysis. The quantity of $G_{(\mathrm{RADA1})}$ is greatest on spleen cells, intermediate on bone marrow and lymph node cells, and least on thymocytes.

marrow and lymph node cells were found to have approximately two-thirds the $G_{(\mathrm{RADA1})}$ quantity of spleen cells, and thymocytes approximately one-fifth the quantity (Fig. 3). This pattern of $G_{(\mathrm{RADA1})}$ in the tissues of young AKR mice is similar to the distribution of G_{IX} and GCSA, the two previously recognized cell surface antigens related to naturally occurring MuLV (1, 4, 5).

STRAIN DISTRIBUTION OF $G_{(\mathrm{RADA1})}$. As spleen was the richest source of $G_{(\mathrm{RADA1})}$ in normal AKR mice, other mouse strains were typed for $G_{(\mathrm{RADA1})}$ by absorption tests with normal spleen. Table I summarizes the strain distribution of $G_{(\mathrm{RADA1})}$ in relation to the G_{IX} and GCSA phenotype of these mice. From this, the following conclusions can be drawn:

(*a*) High leukemia-incidence strains, e.g., AKR, C58, C3H/Figge, and AKR-H-2^b are $G_{(\mathrm{RADA1})}{}^+$. The spleens of these strains also type $G_{IX}{}^+$GCSA$^+$.

(*b*) Low leukemia-incidence strains, e.g., A, C57BL, and BALB/c are $G_{(\mathrm{RADA1})}{}^-$. In these strains, $G_{(\mathrm{RADA1})}$ cannot be detected by absorption tests in any normal tissue.

(*c*) The C3Hf/Bi strain is referred to as a conversion strain because it undergoes a $G_{IX}{}^-$ GCSA$^-$ → $G_{IX}{}^+$GCSA$^+$ change in the lymphoid tissues between 2 and 6 mo of age, and this antigenic conversion is associated with MuLV production (14). $G_{(\mathrm{RADA1})}$ shows a similar pattern in C3Hf/Bi mice, being absent in 2-mo-old mice but present at 6 mo of age.

(*d*) In this survey of inbred strains, $G_{(\mathrm{RADA1})}$ was never found in the absence of GCSA and G_{IX}. However, GCSA and G_{IX} can be expressed in the absence of $G_{(\mathrm{RADA1})}$. For example, 4- to 6-mo-old NZB mice type GCSA$^+G_{IX}{}^+$ but $G_{(\mathrm{RADA1})}{}^-$. (The $G_{IX}{}^+$GCSA$^-G_{(\mathrm{RADA1})}{}^-$ phenotype is characteristic of many low leukemia-incidence strains, e.g., 129, A, etc. In these strains, lymphoid tissues other than thymus do not express G_{IX}, and this contrasts with the presence of G_{IX} in all lymphatic tissues of $G_{IX}{}^+$ high leukemia-incidence mice [4].)

(*e*) Both $G_{(\mathrm{RADA1})}$ and G_{IX} are expressed in F_1 hybrids of AKR with other mouse strains. F_1 expression of GCSA, however, depends in most instances on the *Fv-1*

TABLE I

*Occurrence of $G_{(RADA1)}$ in the Spleen of Inbred and Hybrid Mice: Relation to G_{IX} and GCSA Phenotypes**

$G_{(RADA1)}^+$		$G_{(RADA1)}^-$		
$G_{IX}^+GCSA^+$	$G_{IX}^+GCSA^-$	$G_{IX}^+GCSA^+$	$G_{IX}^+GCSA^-$	$G_{IX}^-GCSA^-$
Inbred mice:				
AKR, AKR/T1		NZB (old)‡	A	129-G_{IX}^-
AKR-H-2^b			129	C57BL
C58			C57BL-G_{IX}^+	C57BR
C3H/Figge			C57BL-G_{IX}^+M	C57L
C3Hf/Bi (old)‡			C57BR-G_{IX}^+M	BALB/c
			I	CBA/T6
			DBA/2	C3Hf/Bi (young)
			SJL/J	RF
			C3H/An	HSFS/N
			NZB (young)‡	
Hybrid mice:				Random bred mice
AKR × C3Hf/Bi§	AKR × CBA/T6			Swiss Ha/ICR
C57L × AKR	AKR × RF			
	C57BL × AKR			

* $G_{(RADA1)}$ and GCSA typing by absorption tests with spleen cells; G_{IX} typing by absorption tests with thymocytes.

‡ Conversion strains (see text).

§ Reciprocal cross tested.

allele contributed by the low leukemia strain partner (1). (*Fv-1* alleles control th consequence of infection by MuLV by permitting or restricting viral replicatio and dissemination [15]. Matings of AKR [genotype *Fv-1^n*] with other Fv-1^n strain e.g., C3Hf/Bi or C57L produce $GCSA^+$ hybrids, whereas matings with Fv-1 strains, e.g., C57BL or with the Fv-1^{nr} RF strain [W. P. Rowe, personal communication] produce $GCSA^-$ hybrids. The notable exception to this rule is the $GCSA^-$ phenotype of AKR hybrids with CBA/T6, an Fv-1^n strain; see reference 1 for discussion.) Thus, the G_{IX}^+ $G_{(RADA1)}^+$ $GCSA^-$ phenotype, which is not seen in any of the parental inbred strains, is unique for hybrids.

PRELEUKEMIC AMPLIFICATION OF $G_{(RADA1)}$ EXPRESSION IN AKR THYMUS. Thymocytes of AKR mice undergo age-related changes in expression of H-2 and Thy-1 alloantigens and MuLV-related antigens such as G_{IX} and GCSA (16). In contrast to the high Thy-1/low H-2, G_{IX}, GCSA surface phenotype of thymocytes of 2-mo-old AKR mice, thymocytes from 6-mo-old AKR mice frequently exhibit a low Thy-1/high H-2, G_{IX}, GCSA phenotype. This change is not associated with increased production of ecotropic MuLV, but correlates closely with the emergence of MuLV with the capacity to grow on mink cells (17, 18). As illustrated in Fig. 4, the expression of $G_{(RADA1)}$ is also amplified in 6-mo-old AKR thymocytes. Parallel tests for G_{IX} and for $G_{(RADA1)}$ on thymocytes from individual AKR donors showed that G_{IX} amplification invariably accompanies $G_{(RADA1)}$ amplification but that G_{IX} can be amplified without amplification of $G_{(RADA1)}$, once again indicating that expression of these two MuLV-related traits is under separate control.

$G_{(RADA1)}$ Phenotype of Tumor Cells. A survey of the $G_{(RADA1)}$, G_{IX}, and GCSA phenotypes of over 15 transplanted mouse tumors is given in Table II. As expected, the two transplanted leukemias (K36 and AKSL2) arising in the $G_{(RADA1)}^+$ AKR strain were $G_{(RADA1)}^+$ as were 11 primary AKR leukemias. $G_{(RADA1)}^+$ tumors occur also in $G_{(RADA1)}^-$ strains (e.g., RADA1, E♂G2, Meth 4), just as $G_{IX}^+GCSA^+$ tumors arise in $G_{IX}^-GCSA^-$ strains. Typing for G_{IX} and

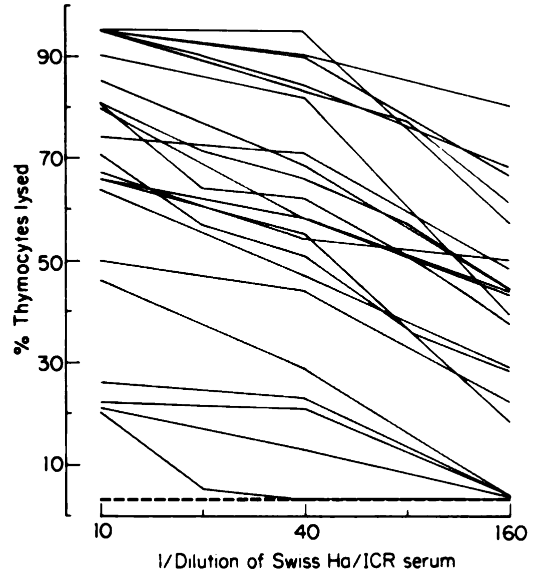

FIG. 4. Amplified $G_{(\mathrm{RADA1})}$ expression by thymocytes from 6-mo-old AKR mice. Each line represents a cytotoxic test with $G_{(\mathrm{RADA1})}$ typing serum and thymocytes from an individual AKR mouse. Dashed line represents cytotoxic tests with thymocytes from 31 6-mo-old AKR mice showing no amplification in $G_{(\mathrm{RADA1})}$ expression. (Thymocytes from 2-mo-old AKR mice are not sensitive to $G_{(\mathrm{RADA1})}$ antibody in direct cytotoxic tests, but by absorption tests can be shown to express $G_{(\mathrm{RADA1})}$.)

$G_{(\mathrm{RADA1})}$ were generally concordant; no $G_{\mathrm{IX}}^{-}G_{(\mathrm{RADA1})}^{+}$ tumor was found but two $G_{\mathrm{IX}}^{+}G_{(\mathrm{RADA1})}^{-}$ leukemias were. The GCSA phenotype was also generally concordant with the $G_{\mathrm{IX}}/G_{(\mathrm{RADA1})}$ phenotype. However, tumors with exceptional $G_{\mathrm{IX}}/G_{(\mathrm{RADA1})}$/GCSA phenotypes have been found, e.g., RADA1, RL♂1, MOPC-70A, and 129RAD4.

Induction of $G_{(\mathrm{RADA1})}$ by MuLV Infection. Analysis of the $G_{(\mathrm{RADA1})}$ system revealed several close parallels with the G_{IX} and GCSA systems. G_{IX}, GCSA, and $G_{(\mathrm{RADA1})}$ occur together in the normal tissues of high leukemia-incidence strains, show amplified expression in thymus of 6-mo-old AKR mice, and appear in the leukemias and solid tumors of strains whose normal tissues lack these antigens. These similarities suggest that $G_{(\mathrm{RADA1})}$, like G_{IX} and GCSA, is specified by MuLV genes. As shown in Table III, $G_{(\mathrm{RADA1})}$ is expressed by permissive cells after in vitro infection by certain isolates of MuLV. $G_{(\mathrm{RADA1})}$ was induced by four of five N-tropic MuLV and by one of four B-tropic MuLV isolates. MuLV with the capacity to infect cells of heterologous species but not cells of the mouse (xenotropic MuLV) do not induce $G_{(\mathrm{RADA1})}$, and this is consistent with the observation that the tissues of NZB mice, a strain that naturally produces high levels of xenotropic MuLV throughout life, types $G_{(\mathrm{RADA1})}^{-}$ (Table I). The prototype MCF MuLV, MCF 247, a virus that is believed to have arisen by a recombinational event between an N-tropic and xenotropic MuLV (18–20), behaves like an N-tropic MuLV rather than a xenotropic MuLV with regard to $G_{(\mathrm{RADA1})}$ induction. A new MCF isolate, AKR MCF 69L1, also behaves in this fashion.

TABLE II

G_{IX}, $G_{(RADA1)}$, and GCSA Phenotypes of Transplanted Tumors of the Mouse

Tumor	Strain of origin	Method of tumor induction and type	MuLV-related cell surface antigens		
			G_{IX}	$G_{(RADA1)}$	GCSA
In vivo					
RADA1	A	X-ray leukemia	+	+	−
ASL1	A	Spontaneous leukemia	−	−	−
E♂G2	C57BL	MuLV-Gross leukemia	+	+	+
ERLD	C57BL	X-ray leukemia	−	−	−
EL4	C57BL	DMBA leukemia	−	−	−
Meth A	BALB/c	Methylcholanthrene sarcoma	−	−	−
RL♂1	BALB/c	X-ray leukemia	+	−	+
MOPC-70A	BALB/c	Mineral oil myeloma	−	−	+
K36	AKR	Spontaneous leukemia	+	+	+
AKSL2	AKR	Spontaneous leukemia	+	+	+
129RAD4	129	X-ray leukemia	+	−	−
129-G_{IX}^--RAD17	129-G_{IX}^-	X-ray leukemia	−	−	−
In vitro					
Meth 4	C57BL	Methylcholanthrene sarcoma	+	+	+
B6MS2	C57BL		+	+	+
Meth A(s)*	BALB/c		+	+	+
Meth A(a)*	BALB/c		−	−	−
CMS3	BALB/c		+	+	+
CMS4	BALB/c		−	−	−
CMS5	BALB/c		−	−	−

* Meth A(s) and Meth A(a) refer to cell lines derived from the parental Meth A sarcoma (s) a the ascites variant derived from it (a); see reference 9.

In this series of tests, no dissociation between G_{IX} and $G_{(RADA1)}$ typing was observed; MuLV induced either the $G_{IX}^+G_{(RADA1)}^+$ or the $G_{IX}^-G_{(RADA1)}^-$ phenotype. No MuLV was found that caused expression of G_{IX} in the absence of $G_{(RADA1)}$ and GCSA, a surface phenotype that is found in vivo on thymocytes (Table I) and on tumor cells (Table II). GCSA is a general marker for MuLV replication, with both N- and B-tropic MuLV and xenotropic MuLV (with the exception of AT124) inducing this antigen in permissive cells (10).

Relation of $G_{(RADA1)}$ to Structural Components of MuLV

CYTOTOXIC TESTS WITH ANTI-MULV-COMPONENT SERA. Fig. 5 shows cytotoxic tests with RADA1 cells and heterologous antisera to MuLV-structural components. RADA1 is lysed by two anti-gp70 sera (anti-MuLV[AKR] gp70 and anti-MuLV[Scripps] gp70) known to have broad gp70 reactivity (E. Fleissner, unpublished data), but not by a type-specific gp70 antisera prepared against MuLV(Rauscher) gp70. Antisera to the p15 and p30 core components of MuLV(AKR) and MuLV(Rauscher) were not cytotoxic for RADA1. This finding is consistent with the GCSA$^-$ phenotype of RADA1. (GCSA is related to the internal core proteins of MuLV, p15, and p30, which occur as glycosylated polyproteins on the surface of infected cells [11]. For this reason, anti-p15 and anti-p30 lyse GCSA$^+$ cells but not GCSA$^-$ cells [1]).

ABSORPTION TESTS WITH MULV STRUCTURAL COMPONENTS. The cytotoxic reactivity of anti-gp70 sera with RADA1 could be directed against the G_{IX}-gp70

TABLE III

Induction of G_{IX}, $G_{(RADA1)}$, and GCSA after Infection by MuLV

MuLV	Host range	MuLV-induced cell surface antigens*		
		G_{IX}	$G_{(RADA1)}$	GCSA
	Ecotropic:			
WN1802N CLB5	N-tropic	++	++	++
WN1802B CLD1	B-tropic	−	−	++
B6(N) CLA3	N-tropic	++	++	++
B6-7(B) CLD3	B-tropic	++	++	+
B6Mai-10(B)‡	B-tropic	−	−	++
BALB:N.3§	N-tropic	−	−	++
BALB:B.6§	B-tropic	−	−	++
AKR-L1 CLG12	N-tropic	++	++	++
AKR 69E5‖	N-tropic	++	++	++
Moloney CLH6	NB-tropic	−	−	+
Rauscher CL1	NB-tropic	−	−	+
S16CL10(I)	Xenotropic	−	−	+
AT124	Xenotropic	−	−	−
NZB	Xenotropic	−	−	+
AKR 69X9‖	Xenotropic	−	−	+
AKR MCF 247¶	Dualtropic	++	++	++
AKR MCF 69L1**	Dualtropic	++	++	++

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* G_{IX}, $G_{(RADA1)}$, and GCSA typing by absorption tests (see Materials and Methods and reference 10). ++, complete absorption; +, partial absorption; and −, no absorption of cytotoxic reactivity from appropriately diluted typing sera.

‡ Isolated from pooled spleen, lymph node, and thymus tissue of a 12-mo-old C57BL/6 Mai mouse (J. W. Hartley, personal communication).

§ Isolated from the spleen of a 16-mo-old BALB/c mouse (P. V. O'Donnell, unpublished data).

‖ Cloned viruses isolated from the thymus of a 6-mo-old AKR mouse (2169) exhibiting amplified expression of MuLV antigens (P. V. O'Donnell, E. Stockert, and L. J. Old, unpublished data).

¶ Reference 18.

** Isolated from a 5-mo-old leukemic AKR mouse injected intrathymically at 2 mo of age with culture fluid from mink cells co-cultured with AKR 2169 thymus tissue (J. A. Lewis, P. V. O'Donnell, E. Stockert, and L. J. Old, unpublished data).

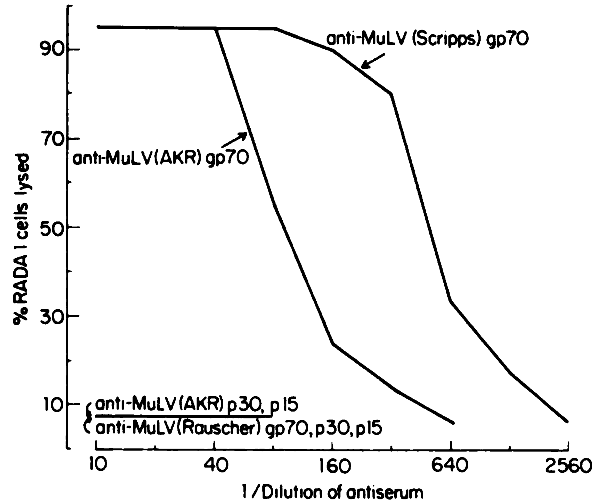

FIG. 5. Cytotoxic tests with RADA1 target cells and heteroantibody to MuLV(Scripps), MuLV(AKR), and MuLV(Rauscher) structural components. Antibody to MuLV(Scripps) gp70 and MuLV(AKR) gp70 cytotoxic for RADA1 cells.

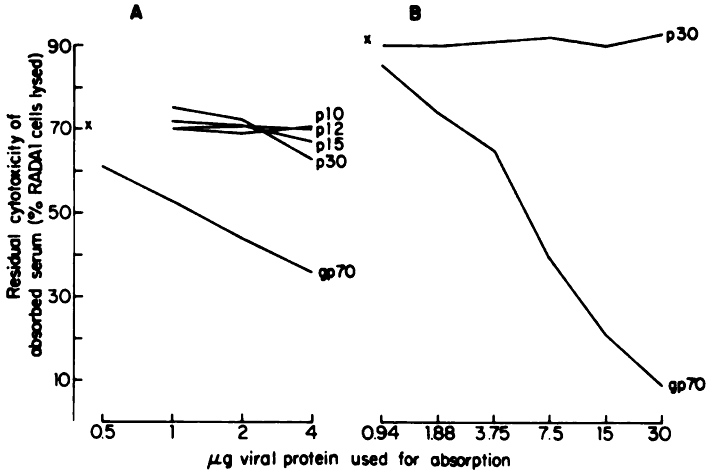

FIG. 6. (A) and (B) Two absorption tests with isolated MuLV(Gross) structural components. RADA1 typing serum (diluted according to end point) is absorbed with graded quantities of viral protein and tested for residual cytotoxic antibody against RADA1 cells. X = percent RADA1 lysed by unabsorbed anti-$G_{(RADA1)}$ serum (diluted 1:25). Absorption of anti-$G_{(RADA1)}$ reactivity is seen with MuLV(Gross) gp70.

expressed by RADA1 or against other gp70 cell surface molecules, possibly gp70 with $G_{(RADA1)}$ determinants. To investigate the relation of $G_{(RADA1)}$ to gp70 more directly, purified MuLV structural components were tested for their capacity to absorb $G_{(RADA1)}$ antibody from the Swiss typing serum (Fig. 6 A and B). Whereas MuLV(Gross) p10, p12, p15, and p30 removed virtually no cytotoxic activity, MuLV(Gross) gp70 absorbs anti-$G_{(RADA1)}$ reactivity in a dose-dependent fashion.

IMMUNOPRECIPITATION TESTS WITH RADIOLABELED MuLV. The presence of antibody to gp70 in the $G_{(RADA1)}$ typing serum was shown in experiments involving precipitation of [^{3}H]amino acid- or [^{3}H]glucosamine-labeled components of MuLV(Gross) by anti-$G_{(RADA1)}$ serum and goat anti-mouse immunoglobulin serum. The only MuLV component precipitated by $G_{(RADA1)}$ antiserum was gp70.

IMMUNOPRECIPITATION TESTS WITH RADIOLABELED RADA1 CELLS. The surface molecules carrying $G_{(RADA1)}$ determinants were characterized by enzymatic radioiodinization of viable RADA1 cells, precipitation with $G_{(RADA1)}$ typing serum, and analysis by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. An electropherogram of the proteins precipitated by anti-$G_{(RADA1)}$ serum is shown in Fig. 7A. Two major protein species were observed. One species co-electrophoresed with an MuLV(Gross) gp70 marker and was shown to be a glycoprotein by the galactose oxidase-sodium-[^{3}H]borohydride method of labeling surface glycoproteins (21). The other species, designated "a", is a nonspecifically precipitated protein which has been identified as actin by its selective precipitation with anti-actin serum (H. W. Snyder, Jr., unpublished data). The precipitating activity of anti-$G_{(RADA1)}$ serum for the 70,000 dalton species on RADA1 cells was not removed after absorption with a $G_{(RADA1)}^-$ cell population (thymocytes from C57BL-G_{IX}^+ mice), but was

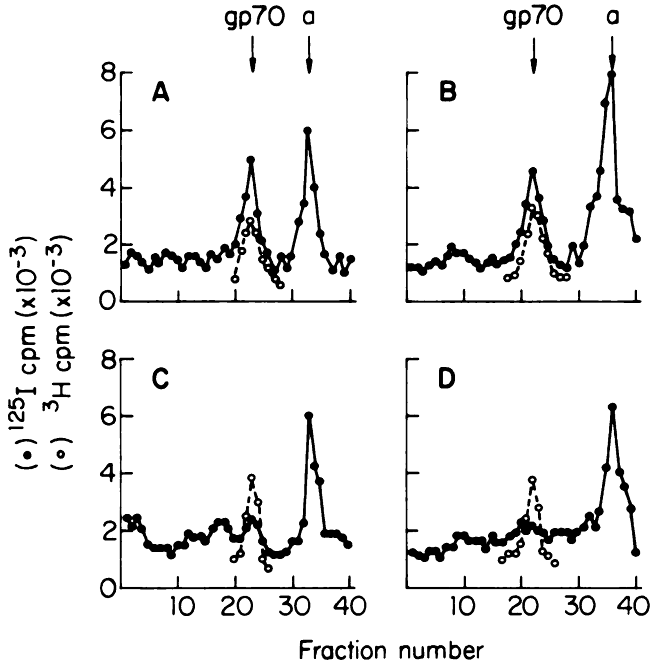

FIG. 7. Co-electrophoresis of [^{3}H]glucosamine-labeled MuLV(Gross) gp70 (○) with ^{125}I-labeled surface proteins of RADA1 leukemia cells (●) precipitated from a cell lysate with: (A) unabsorbed Swiss $G_{(\mathrm{RADA1})}$ typing serum (=anti-$G_{(\mathrm{RADA1})}$); (B) anti-$G_{(\mathrm{RADA1})}$ absorbed with C57BL-G_{IX}^{+} thymocytes (a $G_{(\mathrm{RADA1})}^{-}$ cell population); (C) anti-$G_{(\mathrm{RADA1})}$ absorbed with RADA1 leukemia cells; (D) Swiss mouse serum lacking $G_{(\mathrm{RADA1})}$ antibody.

removed by absorption with RADA1 leukemia cells (Fig. 7B and C). No molecule of this size class was precipitated by the anti-$G_{(\mathrm{RADA1})}$ serum from comparably labeled A strain thymocytes and ASL1 leukemia cells; this finding is consistent with the $G_{(\mathrm{RADA1})}^{-}$ phenotype of these two A strain cells.

Occurrence of Cytotoxic Antibody for RADA1 Cells in Normal Mouse Serum. A preliminary survey of 25 inbred and hybrid strains of mice showed that naturally occurring cytotoxic antibody for RADA1 cells is not restricted to random-bred Swiss mice (Table IV). High titered sera were also found in individual C57BL, A, BALB/c, and C57BL hybrid mice. Cytotoxic (C57BL × A)F$_1$ sera were selected for specificity tests by absorption analysis; reactivity for $G_{(\mathrm{RADA1})}$ was shown to be identical to reference Swiss serum. Sera with reactivity against RADA1 cells were also tested for naturally occurring G_{IX} antibody. As reported previously, anti-G_{IX} is found in F$_1$ hybrids resulting from C57BL and C57BL-G_{IX}^{+} × 129 matings, but not in the parental inbred strains (22). Parallel tests of sera from individual mice showed that G_{IX} and RADA1 reactivity were clearly separable.

Discussion

With the description of the $G_{(\mathrm{RADA1})}$ antigen, four systems of MuLV-related cell surface antigens detected by mouse antibody can now be distinguished. Three of these (GCSA, G_{IX}, and $G_{(\mathrm{RADA1})}$) are specified by naturally occurring

TABLE IV

*Occurrence of Natural Cytotoxic Antibody for RADA1 Cells in Mice of Various Genotypes**

Strain	Total number of mice tested	Cytotoxic test (% RADA1 lysed)				
		≥95	94–75	74–50	49–15	<15
				Number of mice		
Swiss Ha/ICR	82	6	1	6	6	63
129	8					8
129-G_{IX}^-	5					5
C57BL	20	3	3	1	1	12
C57BL-G_{IX}^+	17	1	1	1	2	12
C57BL-G_{IX}^+M	14	2 (2)‡		2 (2)	1	9
C57BR	8				5	3
C57BR-G_{IX}^+M	8				4	4
C57L	8				2	6
C58	4					4
AKR	12					12
AKR-H-2^b	10			2	6	2
DBA/2	9			1	1	7
C3Hf/Bi	6			1	3	2
A	8		1			7
BALB/c	10		1		8	1
SJL/J	4					4
HSFS/N	6					6
HRS/J	9			2		7
NZB	23			4	5	14
C57BL × 129-G_{IX}^-	39			1	15	23
C57BL-G_{IX}^+ × 129	32	2 (2)		9 (6)	15	6
C57BL × A	18	3	4	3	5	3
C57BL-TL^+ × A	21		3 (1)	2	5	11
C57BL × A-TL^-	21	2	2	1	6	10
NZB × NZW	31		1 (1)	1	8	21

* Serum was collected from 6- to 12-mo-old mice, diluted 1:10, and tested individually for cytotoxic activity against RADA1 cells. All sera with reactivity against RADA1 cells (>50% RADA1 lysed) were also tested for naturally occurring G_{IX} antibody in cytotoxic tests with C57BL-G_{IX}^+ thymocytes ($G_{IX}^+G_{(RADA1)}^-$ target cells) and C57BL thymocytes ($G_{IX}^-G_{(RADA1)}^-$ target cells).

‡ Numbers in parentheses refer to proportion of mice with G_{IX} antibody in addition to cytotoxic RADA1 antibody.

endogenous MuLV (1), and the other, the Friend, Moloney and Rauscher (FMR) complex (23), occurs only on cells after exogenous infection with FMR viruses. We are currently analyzing three additional antigens belonging to this general category of cell surface molecules and there is reason to expect that the list will continue to grow. A nomenclature, comparable to the one adopted for structural components of MuLV and other oncornaviruses (24), that would relate these cell surface antigens to MuLV proteins would be highly desirable, and the basis for such a nomenclature has begun with the demonstration that G_{IX} and $G_{(RADA1)}$ are related to gp70 molecules (25, 26) and that GCSA is related to internal virion components, p15 and p30 of MuLV (11). However, until agreement can be reached on an appropriate nomenclature, we propose that new cell surface specificities related to endogenous MuLV follow the $G_{(RADA1)}$ convention; G, as a

TABLE V

G_{IX}, $G_{(RADA1)}$, and GCSA Phenotypes of Mouse Lymphoid Cells in Vivo and MuLV-Infected Cells in Vitro

Phenotype	Occurrence in vivo	In vitro induction by MuLV
$G_{IX}^{+}G_{(RADA1)}^{+}GCSA^{+}$	Normal lymphoid tissues and leukemias of high leukemia-incidence strains (e.g., AKR, C58) that are overt, life-long producers of MuLV	Permissive cells infected by certain ecotropic MuLV and by AKR MCF 247 and 69L1
$G_{IX}^{-}G_{(RADA1)}^{+}GCSA^{+}$	Not found	Not found
$G_{IX}^{+}G_{(RADA1)}^{-}GCSA^{+}$	BALB/c RL♂1 (a BALB/c leukemia induced by X-ray)	Not found
$G_{IX}^{+}G_{(RADA1)}^{+}GCSA^{-}$	RADA1 (an A strain leukemia induced by X-ray)	Not found
$G_{IX}^{-}G_{(RADA1)}^{-}GCSA^{+}$	Leukemias induced by FMR viruses (but otherwise not found in normal lymphoid tissues or in spontaneous leukemias or leukemias induced by X-ray or chemicals); observed in MOPC-70A (a BALB/c myeloma induced by mineral oil)	Permissive cells infected by most B-tropic MuLV, FMR MuLV, and most xenotropic MuLV
$G_{IX}^{-}G_{(RADA1)}^{-}GCSA^{-}$	Normal thymocytes of low leukemia-incidence G_{IX}^{-} strains (e.g., C57BL, BALB/c)	Permissive cells infected by AT124 xenotropic MuLV
$G_{IX}^{+}G_{(RADA1)}^{-}GCSA^{-}$	Normal thymocytes of low leukemia-incidence G_{IX}^{+} strains (e.g., 129, A strain)	Not found
$G_{IX}^{-}G_{(RADA1)}^{+}GCSA^{-}$	Not found	Not found

generic term for naturally occurring MuLV, in honor of Ludwik Gross who discovered murine leukemia viruses, followed by the designation of the prototype normal or malignant cell, e.g., RADA1 used in the definition of the antigenic system. In this light, GCSA would now be renamed $G_{(E_{♂}G2)}$ and G_{IX} would become $G_{(129t)}$ (t = normal thymocytes). Clearly, GCSA and G_{IX} are no longer appropriate designations; the term GCSA could refer to all cell surface antigens related to endogenous MuLV, and the original basis for naming G_{IX}, the assignment of a gene specifying G_{IX} to the IX linkage group of the mouse (4), we now know to be incorrect because the apparent relationship between G_{IX} and linkage group IX is one of pseudo- or quasilinkage and not true linkage (27). However, because of the widespread use of both the GCSA and G_{IX} terminology, it would seem inadvisable to propose changing the designation of these two MuLV-related antigens until a definitive nomenclature can be established.

Table V lists the eight possible G_{IX}, $G_{(RADA1)}$, and GCSA phenotypes and examples of their occurrence on normal and malignant lymphoid cells in vivo or on permissive cells after MuLV infection in vitro. Two in vivo phenotypes have never been observed, and this relates to the fact that $G_{(RADA1)}$ has not been found on cells lacking the G_{IX} trait. G_{IX}, in contrast, can be expressed in the absence of $G_{(RADA1)}$, and the best example of this is its occurrence on the thymocytes of certain low leukemia-incidence strains, such as 129 or A strain mice. Present evidence indicates that both G_{IX} and $G_{(RADA1)}$ are type-specific determinants present on gp70 molecules of certain MuLV, and that in the case of the prototype $G_{(RADA1)}^+$ leukemia RADA1, both antigens reside on the same gp70 molecule. Taken together, this information might suggest that MuLV induction assays would reveal three of four possible $G_{IX}/G_{(RADA1)}$ surface phenotypes (the absent one, $G_{IX}^-G_{(RADA1)}^+$). Only two were actually found ($G_{IX}^-G_{(RADA1)}^-$ and $G_{IX}^+G_{(RADA1)}^+$); the MuLV isolates we examined coded for either both antigens or neither. Attempts are currently underway to isolate a $G_{IX}^+G_{(RADA1)}^-$–inducing MuLV variant from leukemia cells having this phenotype (e.g., BALB/c RL♂1).

A characteristic of G_{IX}, $G_{(RADA1)}$, and GCSA is their appearance in spontaneous or X-ray-induced leukemias of mouse strains that lack these antigens in their normal tissues, and this can be attributed to the derepression or activation of endogenous MuLV either as a cause or consequence of leukemogenesis. Whereas the genes for G_{IX} and GCSA appear to be ubiquitous in mice, as indicated by the occurrence of $G_{IX}^+GCSA^+$ leukemias and solid tumors in varying numbers in the mouse strains tested, too few tumors have been examined to draw the same conclusion for $G_{(RADA1)}$. However, the strain distribution of antibody to $G_{(RADA1)}$ would suggest that $G_{(RADA1)}$ genetic information is widespread in the mouse population. An important consequence of the activation of G_{IX}, $G_{(RADA1)}$, and GCSA genes in malignant cells of strains not normally expressing these antigens is that these new surface components could serve as tumor-specific antigens. The fact that mice can recognize these endogenous MuLV-related cell surface antigens as foreign under certain circumstances and form demonstrable antibody raises the possibility that such immune reactions may have an important, if not determining, role in the spread of virus in the infected host and in the emergence of transformed cells. If appearance of $G_{(RADA1)}$ is a consistent feature of X-ray-induced leukemias in certain mouse strains, it will be of interest to know whether the incidence of these leukemias might be reduced by $G_{(RADA1)}$ antibody, acquired by either passive or active immunization.

Three additional systems of cell surface antigens related to MuLV have now been detected by our laboratory using naturally occurring mouse antibody, and these await detailed analysis. The expectation is that the array of diverse MuLV-related cell surface antigens will parallel the array of distinct MuLV types that exist in the mouse. Current evidence points to the fact that the MuLV family is remarkably polymorphic. The source of this extensive variation is unknown, but clearly recombinational events between classes of MuLV or between MuLV and host genes provide ample opportunity for an almost endless range of MuLV variants to arise. These recombinants could be generated during the lifetime of the host or have arisen in a distant ancestor and then be fixed in the strain as a consequence of stable integration. The array of cell surface antigens coded for by these MuLV variants may turn out to be vast and could explain the perplexing diversity of transplantation antigens found on chemically induced tumors and other tumor types of the mouse (9). Because each of these antigens appears to be unique for individual tumors and shows no cross reaction with any other tumor, it was considered unlikely that MuLV could be responsible for such antigenic variation. With the awareness that MuLV polymorphism may be equally diverse, this possibility will have to be reconsidered.

Summary

A new cell surface antigenic system of the mouse, designated $G_{(RADA1)}$, is described. The antigen is defined by cytotoxic tests with the A strain X-ray-induced leukemia RADA1 and naturally occurring antibody from random-bred Swiss mice and can be distinguished from all other serologically detected cell surface antigens of the mouse. Absorption tests indicate that $G_{(RADA1)}$ is present in the normal lymphatic tissue and leukemias of mouse strains with high spontaneous leukemia-incidence, e.g., AKR, C58, and C3H/Figge. Low leukemia-incidence strains, e.g., C57BL/6, BALB/c, and A lack $G_{(RADA1)}$ in their normal tissues, but a proportion of leukemias and solid tumors arising in these strains are $G_{(RADA1)}^+$. The relation of $G_{(RADA1)}$ to MuLV is shown by $G_{(RADA1)}$ appearance after MuLV infection of permissive cells in vitro; four of five N-tropic MuLV isolates, one of four B-tropic MuLV, and none of four xenotropic MuLV induce $G_{(RADA1)}$. Two MCF MuLV, thought to represent recombinants between N-ecotropic and xenotropic MuLV, also induce $G_{(RADA1)}$. Serological and biochemical characterization indicates that $G_{(RADA1)}$ is a type-specific determinant of the gp70 component of certain MuLV. The presence of natural antibody to RADA1 in various mouse strains and the emergence of $G_{(RADA1)}^+$ leukemias and solid tumors in mice of $G_{(RADA1)}^-$ phenotype suggest widespread occurrence of genetic information coding for this antigen.

We thank Doctors E. Fleissner, W. D. Hardy, Jr., and S. J. Kennel for their gifts of anti-MuLV antisera, Dr. A. B. DeLeo for providing cultured lines of mouse sarcomas, and Miss D. R. Weisfogel for her excellent technical assistance.

Received for publication 29 December 1977.

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IMMUNOREGULATORY CIRCUITS AMONG T-CELL SETS

I. T-Helper Cells Induce Other T-Cell Sets to Exert Feedback Inhibition*

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The T-lymphocyte population is divisible into several subclasses; each subclass possesses a distinctive genetic program which combines information for cell-surface phenotype and function (1). In the mouse, there is evidence that T cells which express the $Thy1^+Ly1^+Ly23^-$ surface phenotype ("Ly1 cells") are programmed for helper $(T_H)^1$ function. In contrast, T cells that express the $Thy1^+Ly1^-Ly23^+$ surface phenotype ("Ly23 cells") are programmed for suppressor (T_S) function (1). Isolation of these two T-cell subclasses in mice depleted of T cells ("B mice") has indicated that each belongs to an independent line or branch of thymus-dependent differentiation (2). A third major T-cell subclass, expressing the surface phenotype $Ly1^+2^+3^+$, can react to antigen and differentiate to $Ly23^+$ cytotoxic effector cells (3), suggesting that this subclass probably contains precursor cells that have acquired receptors for antigen but have not yet become committed to either T_H or $T_{C/S}$ function (3).

These findings, and others, are consistent with the view that functionally distinct T-cell sets carry cell surface components that are invariably associated with particular immunologic function. According to this idea, cells carrying the $Ly1^+Ly23^-$ surface phenotype are programmed for helper and not suppressive activity regardless of external conditions, such as the mode or type of antigen stimulation. To test this hypothesis we have stimulated purified populations of $Ly1^+2^-$ T cells with antigen in vitro, by using conditions devised to induce unselected T cells to express optimal levels of antigen-specific suppressive activity (4). We find that (*a*) stimulation of purified Ly1 cells under these conditions results in the generation of T_H but not T_S activity and (*b*) such hyperimmune Ly1 cells also induce a subset of nonimmune T cells to exert potent suppressive effects upon the antibody response. The surface phenotype of the T-cell set responsible for "feedback" inhibition is described in this study.

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* Supported by U. S. Public Health Service grants AI 13600, AI 12184, AI 10497, and CA 08593.

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[1] *Abbreviations used in this paper:* T-lymphocytes, thymus-derived lymphocytes; T_S, T-suppressor cells; T_H, T-helper cells; T_C, T-cytotoxic cells; SRBC, sheep erythrocytes; HRBC, horse erythrocytes; PFC, plaque-forming cell; TNP, trinitrophenyl.

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Materials and Methods

Mice. C57BL/6 (B6) mice 10–14 wk of age were obtained from The Jackson Laboratory, Bar Harbor, Maine. The congenic lines B6-Ly1^a and By-Ly2^{a}3^a (5), phenotypes Ly1.1,2.2,3.2 and Ly1.2,2.1,3.1, respectively, and B6-Tla^a (6, 7) were produced and supplied by Dr. E. A. Boyse, Memorial Sloan-Kettering Cancer Center, New York.

Production and Use of Antisera. Congenic anti-Thy 1.2, anti-Ly1.2, anti-Ly2.2, and anti-Ly3.2 was prepared as described previously (8). The antiserum (B6 × A-Tla^a) anti-A strain leukemia ASL1, heretofore termed "anti-TL" in reference to its reaction with thymocytes, and herein termed anti-Qa1 in reference to its reaction with peripheral T cells, is described elsewhere (6).

Ly1 cells. Highly purified Ly1 cells were obtained after incubation of 5 × 10^7 spleen cells/ml with anti-Ly2.2 (1:30 final dilution) + anti-Ly3.2 (1:30 final dilution) × $^1/_2$ h at 4°C followed by $^1/_2$ h incubation with selected rabbit C at 37°C as described previously (8). This treatment was repeated to obtain highly purified Ly1 populations. Such "twice-treated" cells were then passed over rabbit anti-Fab-coated Sephadex G-200 columns (9) or nylon wool columns (10). If contamination of the effluent population by Ig$^+$ cells was >3%, the cells were discarded. Controls for specificity of elimination by Ly antisera were performed as previously described (8, 9); controls for Qa1 specificity are shown (Table III).

B cells. Highly purified B cells were obtained by treating spleen cells with anti-Ly1.2,2.2,3.2 and Thy1.2 × $^1/_2$ h at 4°C, followed by exposure to rabbit complement at 37°C according to a previously described protocol (8). Again, this treatment was repeated to insure highly purified populations of B cells. Lack of any residual T-cell activity was determined by the presence or absence of a proliferative response to concanavalin A or a PFC response to SRBC.

T cells. Nonimmune T cells were obtained after anti-Fab column passage rather than nylon wool column passage was routinely used, since passage through nylon wool in some cases resulted in a significant decrease in feedback suppressive activity. In some experiments (Table IV), whole spleen cells were used as a source of nonimmune T cells + B cells.

Antigens. Sheep erythrocytes (SRBC) were obtained from Colorado Serum Co., Denver, Colo. Erythrocytes were conjugated to tri-nitrobenzenesulfonic acid according to the method of Rittenberg and Pratt (11).

In Vitro Stimulation of Lymphoid Populations by SRBC

In vitro stimulation of SRBC-specific T-suppressor cells. 10^7 lymphoid cells (usually highly purified Ly1 cells; see above) were incubated along with 2 × 10^6 SRBC according to the method of Eardley and Gershon (4). At the end of 5 days, the remaining viable lymphoid cells were harvested, washed twice, and in some cases, treated again with anti-Ly2.2, anti-Ly3.2 + C, using the procedure described above for obtaining highly purified Ly1 populations. Various numbers of these stimulated cells were then added to fresh cell cultures containing spleen cells and SRBC (see below).

In vitro primary anti-SRBC responses. A modification of the cell culture technique described initially by Mishell and Dutton (12) was used to generate in vitro primary anti-SRBC PFC responses (13). PFC responses per culture were determined by the Cunningham modification of the Jerne plaque assay as described previously (13). The mean and standard error of PFC were calculated from triplicate cultures.

Results

A Comparison of the Suppressive Effects of Unselected T Cells and Ly1 T Cells after in Vitro Stimulation by SRBC (Table I). Purified Ly1 cells that have been stimulated in vitro with high concentrations of SRBC express no demonstrable suppressive activity when added to fresh cultures containing mixtures of purified nonimmune Ly1 cells, B cells, and sheep erythrocytes (Table I); the addition of similar numbers of SRBC-stimulated unselected T cells resulted in ≅70–95% reduction of the anti-SRBC response. Thus, even after in vitro stimulation by antigen under conditions devised to induce optimal suppressive

TABLE I

Influence of SRBC-Stimulated Ly1 Cells on Primary in Vitro SRBC Responses[*]

	SRBC-stimulated T-cell Population (10^5)	Assay Culture: (4 × 10^6 purified B cells + Nonimmune T-cell population)	Anti-SRBC PFC/culture[‡]	Suppression (%)
Exp 1	None	Ly1 (10^6)	7,300 ± 100	–
	Ly1	Ly1 (10^6)	7,600 ± 660	0
	Unselected	Ly1 (10^6)	350 ± 25	95
Control	None	None	0	–
	Ly1	None	2,500 ± 360	–
	Unselected	None	240 ± 120	–
Exp 2	None	Ly1 (2 × 10^6)	4,600 ± 140	–
	Ly1	Ly1 (2 × 10^6)	6,900 ± 380	0
	Unselected	Ly1 (2 × 10^6)	1,380 ± 130	72
Exp 3	None	Ly1 (10^6) + 10^5 Unselected	2,900 ± 320	–
	Ly1	Ly1 (10^6) + 10^5 Unselected	600 ± 110	79
	Unselected	Ly1 (10^6) + 10^5 Unselected	420 ± 60	85
Exp 4	None	Ly1 (10^6) + 10^5 Unselected	13,600 ± 1,200	–
	Ly1	Ly1 (10^6) + 10^5 Unselected	2,270 ± 160	88
	Unselected	Ly1 (10^6) + 10^5 Unselected	270 ± 40	98
Exp 5	None	Unselected T (10^6)	10,230 ± 1,360	–
	Ly1	Unselected T (10^6)	2,240 ± 380	88
	Unselected	Unselected T (10^6)	530 ± 75	95
Exp 6	None	Unselected T (4 × 10^6)	1,900 ± 200	–
	Ly1	Unselected T (4 × 10^6)	500 ± 60	74

[*] The indicated T-cell populations (Ly1 or unselected) were incubated in vitro with 2 × 10^6 SRBC ×5 days: 10^5 cells were added to assay cultures containing 4 ×10^6 B cells and the indicated nonimmune T-cell populations (see Materials and Methods for details of procedure).

[‡] Mean PFC ± standard error of triplicate SRBC-stimulated assay cultures.

activity by unselected T cells, purified Ly1 cells do not suppress the interaction between nonimmune Ly1 cells and B lymphocytes.

Moreover, addition of SRBC-stimulated Ly1 cells to purified B cells resulted in the induction of a substantial anti-SRBC response; addition of SRBC-stimulated unselected T cells did not (Exp 1: control). Taken together, the above findings show that purified Ly1 cells do not have the capacity (or, more precisely, have lost the differentiative option) of directly suppressing the T$_H$-B interaction: Ly1 cells are programmed to induce B cells to secrete antibody.

SRBC-Activated Ly1 Cells can Induce Ly2$^+$ Cells to Exert Feedback Suppressive Effects (Table I). One conclusion drawn from the above experiments was that antigen-stimulated Ly1 cells induce B cells to differentiate to antibody-forming cells (but do not directly suppress Ly1/B-cell interactions). We then asked whether antigen-stimulated Ly1 cells might induce *other* sets of T lymphocytes, including Ly2$^+$ cells, to develop immunoregulatory activity. We observed that the addition of small numbers of unselected nonimmune T cells to fresh cultures containing SRBC-immune Ly1 cells + nonimmune Ly1 cells (identical to the culture populations of Exp 1 and 2) resulted in ≅80–90% reduction of the SRBC response (Table I, Exp 3 and 4). This reduction was noted when as few as 10^5 unselected nonimmune T cells were deliberately added to assay cultures containing SRBC-stimulated Lyl cells + B cells (Exp 3, 4). In four separate experiments, addition of unselected nonimmune T cells to cultures containing small numbers of SRBC stimulated Lyl cells resulted in ~75–90% inhibition of the anti-SRBC response (Exp 1, 2 vs. 3–6).

These findings suggest that signals from in vitro activated Lyl cells can induce nonimmune T-cell populations containing $Ly2^+$ cells (i.e., cells sensitive to anti-Ly2 + C) to exert potent feedback suppressive effects. Additional experiments, in which graded doses of SRBC-stimulated Lyl cells were added to cultures containing a fixed number of nonimmune $Ly2^+$ cells showed that the level of suppression was directly proportional to the numbers of SRBC-stimulated Lyl cells added to the cultures (Fig. 1); i.e., the degree of feedback suppressive activity exerted by a fixed number of nonimmune $Ly2^+$ cells increased in direct proportion to the level of SRBC-activated T_H activity added to the assay cultures.

After in Vivo Immunization by SRBC, Lyl Cells Induce Feedback Inhibition by Nonimmune $Ly123^+$ Cells (Table II; Fig. 2). The data above indicate that Lyl cells that have been exposed in vitro to high concentrations of SRBC (*a*) induce purified B cells to secrete anti-SRBC antibody, and (*b*) induce nonimmune $Ly2^+$ cells to express substantial suppressive effects. To test whether the activities of in vitro stimulated Lyl cells reflect cellular mechanisms governing the magnitude of the antibody response in vivo, we examined the influence of Lyl cells obtained from mice immunized 1–4 wk previously with high concentrations (10^8) of SRBC upon in vitro primary responses to SRBC (Table II). Such isolated Lyl cells, combined with purified B cells, produced substantial SRBC responses (group A). Addition of nonimmune T cells to these cultures resulted in a substantial reduction of the anti-SRBC PFC response (group B). No inhibition was seen when the nonimmune T-cell population was depleted of cells of the $Ly123^+$ subclass (group C).

These findings indicate that (*a*) in vivo immunization procedures also lead to the formation of Lyl cells that can induce nonimmune T cells to exert feedback suppressive effects, and (*b*) the surface phenotype of cells responsible for feedback suppressive activity in the nonimmune T-cell population is $Lyl^+2^+3^+$.

Finally, feedback inhibitory effects exerted by a fixed number of $Ly123^+$ nonimmune T cells could not be overcome by increasing the number of SRBC-immune Lyl cells over a 10-fold range (Fig. 2), again indicating the potency of $Ly123^+$ feedback inhibition (Table I) and confirming the conclusion drawn from analysis of the effects of in vitro stimulated Lyl cells: the level of $Ly2^+$ feedback inhibition increases in proportion to the amount of Lyl T_H signal in the cultures (see Fig. 1).

Cells Mediating Feedback Suppression also Express Surface Qal (Table III). To further delineate the surface phenotype of nonimmune cells responsible for feedback suppression, we asked whether such cells express Qal locus products. Qal surface components are coded for by gene(s) mapping between H2-D and Tla; these components are expressed selectively on a subset of T cells (6). We examined the effects of anti-Qal + C upon feedback suppressive activity

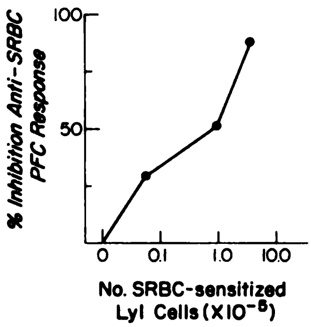

FIG. 1. Increasing numbers of SRBC-sensitized Ly1 cells induce increasing amounts of feedback inhibition by nonimmune T-cells. Increasing numbers of Ly1 cells that had been incubated in vitro 5 days with 2 × 10^6 SRBC were added to the following SRBC-stimulated assay cultures: population A: 5 × 10^6 B-cells + 10^6 nonimmune Ly1 cells + 3 × 10^6 SRBC, or population B: population A + 2 × 10^5 nonimmune T-cells. The percent inhibition of the anti-SRBC response was determined by the following calculation:

$$\frac{\alpha\text{-SRBC PFC response (A)} - \alpha\text{-SRBC PFC response (B)}}{\alpha\text{-SRBC PFC response (A).}}$$

Each point represents the mean percent inhibition of two to four separate experiments.

TABLE II

*After in Vivo Immunization Ly1 Cells can Induce Feedback Inhibition by Nonimmune Ly123$^+$ T-Cells**

	Group	Assay population: SRBC-immune Ly1 cells (1 × 10^5)	Assay population: B cells (4 × 10^6)	Nonimmune T-cell Population Added (10^6)	Anti-SRBC PFC Response: Exp 1	Anti-SRBC PFC Response: Exp 2	Anti-SRBC PFC Response: Exp 3	% Inhibition PFC Response: Exp 1	% Inhibition PFC Response: Exp 2	% Inhibition PFC Response: Exp 3
Experimental cultures	A	+	+	None	9,400	970	1,200	—	—	—
	B	+	+	Unselected	970	225	385	89	83	83
	C	+	+	(Ly1 + Ly23)	9,100	1,925	1,320	2	0	0
Control cultures	D	−	+	Unselected	4,100	750	900			
	E	−	+	None	0	50	0			
	F	+	−	None	0	0	0			

* 10^5 Ly1 cells from donors immunized with 10^9 SRBC i.v. 5–17 days earlier were combined with 4 × 10^6 purified B-cells (obtained after incubation of nonimmune spleen cells with anti-Thy1 + anti-Ly + C; see Materials and Methods). The suppressive effects of different nonimmune T-cell sets upon the generation of anti-SRBC PFC are indicated. Percent inhibition was determined by the following calculation:

$$\frac{\text{PFC group A} - \text{PFC group B (or group C)}}{\text{PFC group A}}$$

of nonimmune spleen cells from a congenic pair of B6 mouse strains differing only at the Qa1 locus (B6-T1^a and B6[T1a$^-$]). Again, addition of SRBC-immune Ly1 cells to nonimmune cell populations lacking Ly123 cells induced substantial SRBC PFC responses; addition of Ly1 cells to unselected spleen cells (containing Ly123 cells) did not (groups A and B). Addition of Ly1 cells to spleen cells treated with anti-Qa1 + C also produced substantial responses (group C). Removal of feedback suppression after treatment with anti-Qa1 + C reflected specific elimination of Qa1 + C cells since treatment of nonimmune cells from

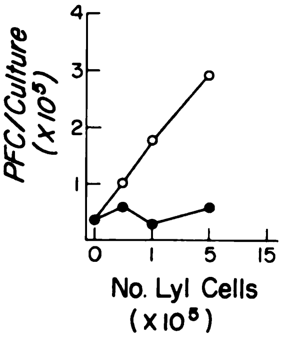

FIG. 2. PFC responses of SRBC-stimulated cell cultures containing graded numbers of SRBC-immune Lyl cells (abscissa) and either 6 × 10^6 normal (NMS + C treated) spleen cells (●) or 6 × 10^6 spleen cells depleted of Lyl23 cells (○) (i.e., mixtures of 3 × 10^6 α-Ly2 + C-treated spleen cells + 3 × 10^6 α-Lyl + C-treated spleen cells). Each point represents the mean PFC of cultures from three separate experiments; in no case was the standard error of the mean greater than 43%.

TABLE III

Feedback Suppression is Mediated by Qal$^+$Lyl23$^+$ T Cells

	Group	SRBC-Immune Lyl Cells (2 × 10^5)	Nonimmune population		Anti-SRBC PFC/culture	
			T Cells (2 × 10^6)	B Cells (4 × 10^6)	Exp 1	Exp 2
Experimental cultures	A	+	Lyl + Ly23	+	2,835	ND
	B	+	Unselected	+	1,065	250
	C	+	Qal$^-$	+	2,510	1,150
	D	+	Qal specificity control*	+	910	280
	E	+	Qal$^-$ + (Lyl + Ly23)	+	2,950	ND
Control cultures	F	+	–	–	0	0
	G	–	Unselected	+	915	600
	H	–	–	+	50	0

* B6(Tla$^-$) Lyl cells added to B6(Tla$^-$) cells that had been treated with anti-Qal + C.

B6-Tla$^+$ donors but not B6(Tla$^-$) donors effectively decreased feedback suppressive activity (group D). The formal possibility that feedback suppression reflected an obligatory interaction between two separate cells, one Lyl23$^+$Qal$^-$ and the other Qal$^+$Lyl23$^-$, is unlikely since mixtures of anti-Ly + C treated cells (containing the putative Qal$^+$Ly$^-$ population) and anti-Qal + C treated cells (containing the putative subset of Lyl23$^+$Qal$^-$ cells) did not exert detectable feedback suppressive effects (group E).

Carrier-Reactive T_H Cells are Targets of Feedback Suppression (Table IV). Feedback suppression by Lyl23 cells might reflect a direct inhibition of induction of T_H cells, delivery of the T_H message, or direct suppression of B-cell differentiation to antibody-forming cells. To address this question, we exploited the observation of Kettman (14) that the development of anti-TNP PFC in this system depends upon the presence of SRBC-immune (carrier-primed) T-helper cells. Therefore, SRBC-immune Lyl cells were added to cultures stimulated

TABLE IV

Feedback Inhibition in Cultures Stimulated with TNP-SRBC

SRBC-Immune Lyl cells (1×10^5)	Nonimmune T cells (2×10^6)	B cells (5×10^6)	Anti-TNP-PFC	Anti-SRBC-PFC
−	Unselected	+	75 ± 30	1,000 ± 75
+	Unselected	+	250 ± 65	700 ± 90
+	(Lyl + Ly23)	+	1,925 ± 410	7,100 ± 440
−	−	+	0	50 ± 8
+	−	−	0	0

The indicated lymphoid cell populations were incubated 5 days in 1-ml cultures containing 3×10^6 TNP-SRBC. PFC responses/culture were determined against SRBC and TNP-HRBC. Background PFC to HRBC were invariably <100 PFC/culture.

with TNP-SRBC conjugates. The presence of Ly123 cells in the nonimmune T-cell population resulted in a marked inhibition of *both* the anti-TNP PFC responses as well as the anti-SRBC response in this system. This finding suggests that feedback suppression reflects, at least in part, inhibition of T_H activity.

Discussion

These experiments were initially designed to determine whether cells carrying the Lyl^+2^- surface phenotype are programmed for helper activity regardless of external conditions, such as the mode or type of antigen stimulation. We have found that in vitro stimulation of purified populations of Lyl cells with sheep erythrocytes, using culture conditions devised to induce optimal T-suppressive (T_S) activity (4), invariably results in the generation of SRBC-specific T helper (T_H) but not T_S activity. These findings indicate that the $Lyl.2^+2.2/3.2^-$ surface phenotype is a stable, invariant marker of T cells that are programmed to express only helper activity and have lost the capacity to directly suppress the antibody response. In general, they support the view that the genetic program for a single differentiated set of cells combines information for surface phenotype and function.

In the course of these studies, we found that highly purified, antigen-stimulated Lyl cells, in addition to inducing B cells to secrete antibody, can induce or activate other sets of T cells to express substantial suppressive effects. The surface phenotype of the set of cells exerting such feedback suppression in these experiments is: $Lyl^+2^+3^+Qal^+$ (referred to below as Ly123 cells for ease of discussion). It is important to note that the Ly123 cell population responsible for feedback suppression was surprisingly resistant to a single treatment with anti-Thyl + C (data not shown). We are presently testing two explanations for this observation: (*a*) Treatment with most anti-Thyl antisera + C eliminates the large majority, but not all, T lymphocytes, or (*b*) Feedback regulatory cells expressing the $Ly123^+Qal^+$ phenotype express relatively low surface concentrations of Thyl and are thus relatively insensitive to lysis by Thyl antiserum + C.

Perhaps the most striking characteristic of Ly123 feedback suppression is the ability of extremely small numbers of these cells to exert potent feedback inhibitory effects in the fact of relatively large numbers of SRBC-immune Lyl cells: fewer than 10^5 nonimmune Ly123 cells were capable of inhibiting ~80–90% of the PFC response induced by 10^6 Lyl cells (Table I). This inhibition was not overcome by increasing numbers of SRBC-immune Lyl cells; in fact, the degree of Ly123-mediated inhibition increased in direct proportion to the level of T_H activity in the system (Fig. 1).

The potency of extremely small numbers of $Ly2^+$ cells in this system is reminiscent of the interaction between Lyl and $Ly2^+$ cells observed during the in vitro generation of alloreactive cytotoxic effector activity (15). In these experiments, extremely small numbers of Ly23 cells generated substantial levels of alloreactive cytotoxic effector activity only if co-cultured with relatively large numbers of Lyl cells. Similarly, the present experiments indicate that extremely small numbers of $Ly2^+$ cells, in this case cells expressing the $Ly123^+Qa1^+$ phenotype, can be induced to express substantial levels of T-suppressive activity in the presence of relatively large numbers of SRBC-immune Lyl cells.

Previous experiments have demonstrated that Lyl cells can (*a*) induce B-cells to produce antibody, (*b*) induce pre-killer cells to differentiate to killer-effector cells, and (*c*) induce monocytes and macrophages to participate in inflammatory reactions (1). As demonstrated in the present experiments, Lyl cells also induce other sets of T cells to develop potent suppressive activity. These findings, taken together, indicate that cells of the Lyl set are programmed to signal other sets of cells to fulfill their respective genetic programs. The present experiments also imply that, like the formation of antibody, the generation of immunologic suppression after stimulation by antigen is not an autonomous function: both require induction by Lyl cells.

The specificity of the feedback effects described here have not been studied, although it is likely that at least one target of suppression is the T_H cell or delivery of the T_H product (Table IV). The problem of specificity can be summed up as follows: although induction of the Ly123-mediated suppression may reflect signals from specifically activated immune Lyl cells, we do not know whether the target of feedback suppression is solely the relevant antigen-reactive T-helper cell (or its product). If so, is such specificity due to Ly123 cells bearing receptors for the antigen or receptors specific for idiotypic determinants carried on the immune Lyl population? Resolution of this question is now in progress using more well-defined antigens that elicit antibodies which carry identifiable idiotypic markers (16).

The magnitude and duration of an antibody response is governed by a complex series of inductive and suppressive interactions among subsets of lymphocytes and macrophages. To delineate these interactions, it is essential to establish whether expression of a particular surface phenotype is a reliable indicator of a cell population's helper-suppressor potential. Demonstration that cells expressing the Lyl^+23^- surface phenotype carry help but are unable to directly suppress, even after antigen stimulation under "suppressive" conditions, allowed dissection of the cellular basis of feedback inhibition by $Ly123^+$ T-cells.

These experiments also bear on current strategies used to define and characterize T-suppressor cells. The observation that addition of cell population X to a complex population of lymphoid cells results in a reduction of the response does not imply that population X contains suppressor cells; it indicates only that the cell population in question can induce a suppressive effect. An apt analogy is that the production of antibody by T-depleted mice after the addition of thymocytes does not necessarily imply that thymocytes produce antibody.

In summary, our results are consistent with the current immunological paradigm that Ly1$^+$23$^-$ T-cells act as obligatory helper (T_H) cells and cannot be induced to act directly as suppressor cells, even by modes of immunization which induce high levels of T_S activity. In addition, the data indicate that, after immunization, Ly1 T_H cells can induce cells expressing the Ly123$^+$Qa1$^+$ phenotype to exert potent inhibitory effects. These findings suggest that activation of resting Ly123 cells by immune Ly1 T_H cells may represent an important homeostatic immunoregulatory mechanism in vivo. We test this proposition in the following paper (17).

Summary

These experiments test the hypothesis that cells carrying the Ly1$^+$23$^-$ surface phenotype are programmed exclusively for helper and not suppressive activity regardless of external conditions such as the mode or type of antigen stimulation. To this end, we have stimulated purified populations of Ly1 cells with antigen in vitro using conditions devised to induce unselected T cells to express optimal levels of antigen specific T-suppressor activity. We find that after such stimulation, Ly1 cells generate SRBC-specific T-helper activity but *not* T-suppressive activity. These findings establish that the *Ly1.2*$^+$,*2.2/3.2*$^-$ surface phenotype is a stable, and probably invariant, marker of T cells that are programmed to express only helper activity and have lost the capacity to directly suppress the antibody response. These findings support the concept that the genetic program for a single differentiated set of cells combines information for cell surface phenotype and function.

We also demonstrate that antigen-stimulated Ly1 cells, in addition to inducing B cells to secrete antibody, can induce or activate other sets of resting T cells to develop profound suppressive effects. The surface phenotype of this feedback suppressive T-cell set is shown to be: Ly1$^+$2$^+$3$^+$Qa1$^+$. These findings, taken together, indicate that activation of resting Ly123 cells by immune Ly1 T_H cells may represent an important homeostatic immunoregulatory mechanism.

Received for publication 29 December 1977.

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IMMUNOREGULATORY CIRCUITS AMONG T-CELL SETS

II. Physiologic Role of Feedback Inhibition In Vivo: Absence in NZB Mice*

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There is increasing evidence that the immune response is regulated by a series of interactions among distinct T-lymphocyte subclasses (1, 2). In the preceding paper we have analyzed one such regulatory interaction: antigen-activated Lyl helper cells induce a second nonimmune set of T cells (cell surface phenotype Lyl^{+}2^{+}3^{+}Qal^{+}) to exert potent "feedback" inhibitory effects which are directly proportional to the level of antigen-induced T-helper activity (3). This T-T interaction was defined by using an in vitro antibody response as a model system, since isolation of different lymphocyte sets is achieved most readily under such in vitro conditions. However, we do not know whether this in vitro T-T interaction (or, for that matter, any cellular interaction that may regulate in vitro antibody responses) plays a physiologic role in the regulation of in vivo immune responses.

We have therefore examined the influence of Ly123^{+} T cells upon in vivo antibody responses. We find that mice lacking such cells do not exhibit feedback suppression, and that the provision of Ly123^{+} T cells to such mice restores this activity. In addition, the relevance of feedback suppression to the physiology of the immune system is underlined by the finding that development of autoimmunity in NZB mice is associated with (*a*) absence of feedback suppression and (*b*) a defect in the differentiation and/or function of T cells expressing the Ly123^{+} phenotype.

Materials and Methods

Animals. C57BL/6 (B6) mice and Ly congenic stock were obtained as described previously (3). NZB and BALB/c mice were bred by H. Cantor from breeders supplied by The Jackson Laboratory, Bar Harbor, Maine.

Production and Use of LY and Thyl.2 Antisera. Preparation and use of Ly-1.2, Ly-2.2, Ly-3.2 and Thy-1.2 antisera have been described (3, 4). The proportion of T cells expressing one or more Ly components was estimated from the lytic effects after sequential exposure to two different Ly antisera with selected rabbit sera as a source of complement (C) according to a protocol detailed

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* Supported by U. S. Public Health Service grants AI 13600, A AI 12184, AI 10497, and CA 08593.

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previously (5). An additional specificity control was employed to rule out the possibility that a portion of lysis seen after treatment of NZB spleen cells with anti-Lyl.2 + C reflected anti-natural killer[1] (NK) reactivity, an additional antibody that has been shown to be present in this serum (6): Ly-1.2 antisera (1:10 dilution) was absorbed with equal volumes of spleen and lymph node cells from B6-Ly-1.1 (NK^+) congenic mice. This serum had no effect on NK activity mediated by NZB or B6 spleen cells and did not exert detectable lytic activity (above normal mouse serum [NMS] + C) against lymph node, spleen, or thymocyte populations from B6-Lyl.1 congenic donors.

Production of "B" Mice. B6 mice were thymectomized at 6 wk of age, lethally irradiated (750 rads; ^{137}cesium source) at 8 wk of age, followed immediately by intravenous infusion of 5 × 10^6 bone marrow cells that had been doubly treated with anti-Thyl + C, according to reference (3). Adult thymectomy (ATx) was also performed at 6 wk of age according to a method described previously (7).

Cellular Repopulation of Irradiated or Nonirradiated Adoptive Hosts. Highly purified Lyl cells from mice immunized with 10^8 sheep erythrocytes (SRBC) 10–30 days previously were obtained according to reference (3) and 5 × 10^5 were injected intravenously along with 10^6 SRBC into irradiated (750 rads) or unirradiated syngeneic recipients. In some cases, nonimmune syngeneic spleen cells or unselected T cells (3) were added to the inocula. Anti-SRBC plaque-forming cell (PFC) responses were measured 5 days later.

Results

A Subset of $Lyl^+2^+3^+$ Cells Exerts Feedback Suppressive Effect during in Vivo Anti-SRBC Responses (Tables I, II). B6 mice depleted of T cells ("B-mice"—see Materials and Methods for details of preparation) did not produce significant anti-SRBC PFC responses after challenge with 10^6 SRBC (Table I, group A). Recipients repopulated with 0.3 × 10^6 Lyl cells from syngeneic donors immunized to SRBC 1 wk previously produced substantial numbers of anti-SRBC PFC 5 days after immunization with 10^6 SRBC (group B). To determine whether a population of nonimmune T cells might exert feedback suppressive effects in vivo, an additional group of B mice was repopulated with nonimmune unselected T cells as well as SRBC-immune Lyl cells. The anti-SRBC PFC response of these mice was reduced to almost that of unrepopulated B-mice (group C). Since B mice repopulated with nonimmune T cells were capable of producing substantial anti-SRBC responses (group D), these findings are consistent with the hypothesis drawn from similar experiments in vitro (3): highly purified, antigen-stimulated Lyl cells induce nonimmune T cells to exert substantial feedback suppressive effects. Finally, elimination of $Lyl23^+$ cells from the nonimmune T-cell population abolished suppression (group E), indicating that the surface phenotype of cells responsible for feedback suppression in vivo is the same as that ascertained in vitro (3). Identical conclusions are drawn from experiments in which lethally-irradiated hosts (rather than B mice) were repopulated with different T-cell sets together with purified B cells (Table II).

Biologic Properties of Feedback Suppressor Cells in Vivo

FEEDBACK SUPPRESSOR CELLS ARE SENSITIVE TO SMALL DOSES OF CYCLOPHOSPHAMIDE IN VIVO (Fig. 1). The PFC responses of mice given 10 mg/kg of cyclophosphamide 24 h before SRBC immunization did not differ significantly

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[1] *Abbreviations used in this paper:* B6, C57BL/6; C, complement; NK, natural killer; Lyl cells, Lyl^+23^- cells; Ly23 cells, Lyl^-23^+ cells; ATx adult thymectomy; NMS, normal mouse serum; NTA, naturally occurring thymocyte autoantibody; SRBC, sheep erythrocytes.

TABLE I

Demonstration of Feedback Suppression by Nonimmune Ly123 Cells in B Mice Repopulated with T-Cell Subclasses

	Cellular repopulation of B mice:			PFC/Spleen*	
	SRBC-Immune Ly1 (0.3 × 10^6)	Nonimmune T-cells (8 × 10^6)	PFC/10^6 Cells	Mean	Range
A.	–	–	8	970	(150-2,100)
B.	+	–	145	12,100	(6,000-16,500)
C.	+	Unselected	22	1,760	(400- 2,600)
D.	–	Unselected	361	31,600	(19,050-40,100)
E.	+	(Ly1 + Ly23)	155	14,650	(10,500-33,600)

* Three to four recipients/group. Mixtures of 8 × 10^6 nonimmune Ly1 + Ly23 cells were obtained by combining 4 × 10^6 anti-Ly1.2 + C-treated T cells with 4 × 10^6 anti-Ly2.2 + C-treated T cells.

TABLE II

Demonstration of Feedback Suppression by Ly123 Cells in Lethally Irradiated Hosts

Cellular repopulation of lethally irradiated hosts:		Anti-SRBC PFC Responses*	
SRBC-Immune T-cells + nonimmune B-cells	Nonimmune T-cells (10^7)	PFC/10^6 Cells (Mean)	PFC/Spleen (Mean and Range)
SRBC-Immune Ly1 (10^6) + 5 × 10^6 B cells +	None	310	1,155 (1,079-1,574)
	Unselected	24	276 (83-498)
	(Ly1 + Ly23)	560	1,245 (950-1,555)

* Three to five recipients/group.

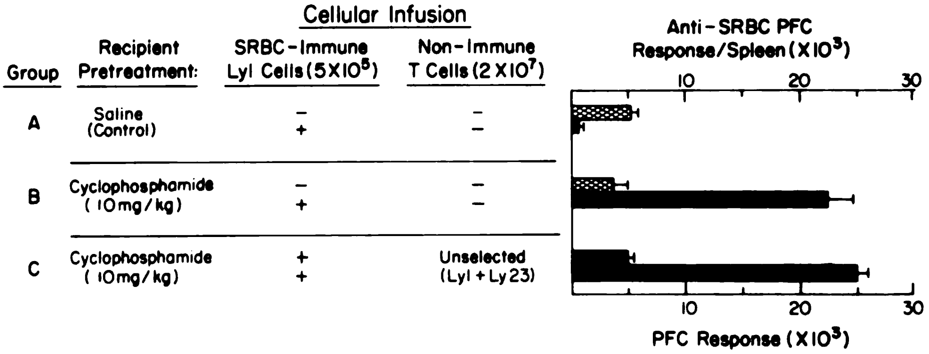


FIG. 1. Ly123 cells mediating feedback inhibition are sensitive to small doses of cyclophosphamide in vivo (see Materials and Methods). The indicated cell populations were injected intravenously along with 2 × 10^6 SRBC into 8–10 wk old syngeneic B6 mice that had been given saline or cyclophosphamide (10 mg/kg i.p.) 24-36 h previously. Purified "SRBC-immune" Ly1 cells (see Materials and Methods for preparation) were obtained from B6 donors immunized with 2 × 10^8 SRBC i.v. 10-30 days previously. The mean and SE of the α-SRBC PFC response of each group (three to five mice/group) was determined 5 days after injection of SRBC. Similar results were obtained in three additional experiments.

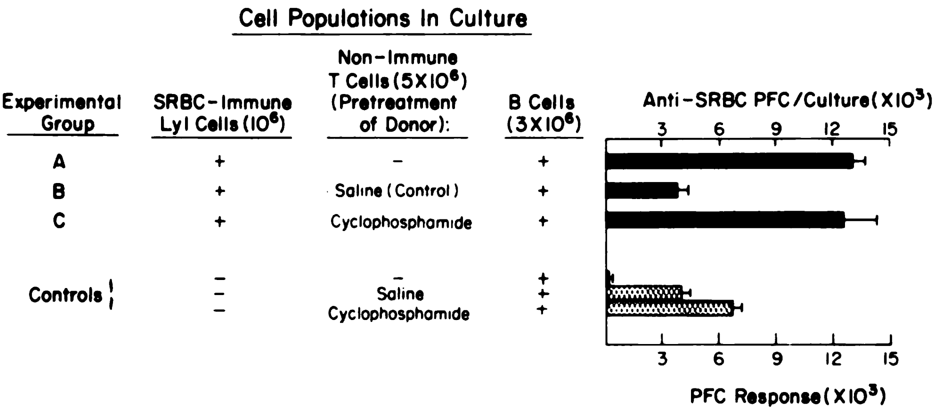
from untreated controls. Adoptive transfer of SRBC-immune Ly1 cells to untreated control mice resulted in over a 90% reduction of the anti-SRBC PFC response (Fig. 1, group A). By contrast, adoptive transfer of identical populations of SRBC-immune Ly1 cells to recipients that had been pretreated with 10 mg/kg cyclophosphamide i.p. 1 day previously resulted in a substantial increase (four to sixfold) in the anti-SRBC PFC response (group B). Thus, the adoptive transfer of purified SRBC-immune Ly1 T-helper cells into normal, untreated hosts failed to increase the host anti-SRBC response; in fact, it resulted in a profound reduction of the response which was associated with the activation of a cyclophosphamide-sensitive population of host cells.

Since the phenotype of nonimmune T cells responsible for feedback suppression in vitro and in vivo (Tables I and II) was $Ly1^+2^+3^+$, we asked whether the same cell population was sensitive to in vivo administration of small doses of cyclophosphamide (10 mg/kg i.p.). SRBC-immune Ly1 cells were inoculated into recipients that had been pretreated with cyclophosphamide on day −2 and repopulated with unselected spleen cells on day −1. Such repopulation reversed the effects of cyclophosphamide: i.e., SRBC-immune Ly1 cells induced potent feedback suppressive effects in spleen cell-repopulated hosts. If $Ly123^+$ cells had been removed from the nonimmune lymphoid population, feedback suppression was abolished (Fig. 1, group C).

Finally, we tested whether the set of cyclophosphamide-sensitive $Ly123^+$ cells responsible for feedback suppression in vivo was also responsible for in vitro feedback effects described previously (3) (Fig. 2): SRBC-immune Ly1 cells were added to culture containing purified B-cells alone (group A), B cells combined with nonimmune T cells from untreated donors (group B), or donors given 10 mg/kg cyclophosphamide i.p. 1 day previously (group C). Feedback suppression was noted only in group B cultures.

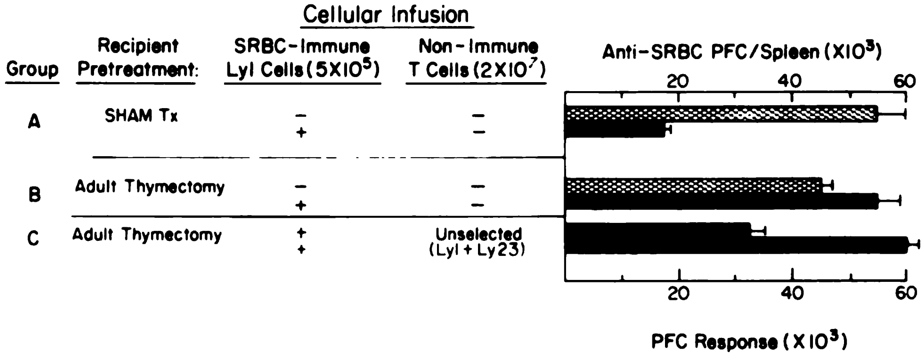
IN VIVO FEEDBACK SUPPRESSIVE ACTIVITY IS REDUCED SHORTLY AFTER ADULT THYMECTOMY (Fig. 3). Since at least a portion of cells belonging to the Ly123 subclass are sensitive to the short-term effects of adult thymectomy (5), we examined the effects of adult thymectomy upon in vivo feedback suppressive activity (Fig. 3). Infusion of SRBC-immune Ly1 cells into animals that had been sham-thymectomized 6 wk earlier induced strong feedback suppressive effects (group A). By contrast, infusion of SRBC-immune Ly1 cells into mice thymectomized 6 wk previously resulted in slightly enhanced responses (group B): and this loss of feedback suppression was regained if adult thymectomized animals were reconstituted with syngeneic spleen cell populations containing cells of the Ly123 subclass (group C).

Precocious Decline of Ly123 Cells and Feedback Suppressive Activity in NZB Mice. NZB mice spontaneously develop an autoimmune disorder characterized by the production of a variety of autoantibodies and a clinical syndrome resembling human systemic lupus erythematosus (8, 9). The suggestion has been put forward that this disorder is due, in part, to abnormal thymus-dependent differentiation, resulting in an imbalance between T-helper cells and T-suppressor cells (8, 9). To examine this question, we first determined proportions of Ly^+ T-cell subclasses in the spleens of NZB and BALB/c mice during the first 12 mo of life. These studies clearly show that during this time



	Cell Populations In Culture		
Experimental Group	SRBC-Immune Lyl Cells (10^6)	Non-Immune T Cells ($5X10^6$) (Pretreatment of Donor):	B Cells ($3X10^6$)
A	+	−	+
B	+	Saline (Control)	+
C	+	Cyclophosphamide	+
Controls	−	−	+
	−	Saline	+
	−	Cyclophosphamide	+

FIG. 2. Nonimmune T cells exerting feedback inhibition in vitro are sensitive to cyclophosphamide. Cultures containing the indicated cell populations were stimulated with 3 × 10^6 SRBC (3) ×5 days. Cyclophosphamide (10 mg/kg) was administered i.p. 24–36 h before sacrifice and peparation of nonimmune spleen cells; purified Lyl cells and purified B cells were obtained as described previously (3). The mean α-SRBC PFC and standard errors for each group are based on the PFC responses of triplicate cultures. Similar results were obtained in four additional experiments.



		Cellular Infusion	
Group	Recipient Pretreatment:	SRBC-Immune Lyl Cells($5X10^5$)	Non-Immune T Cells($2X10^7$)
A	SHAM Tx	−	−
		+	−
B	Adult Thymectomy	−	−
		+	−
C	Adult Thymectomy	+	Unselected (Lyl + Ly23)
		+	

FIG. 3. Ly123 cells mediating feedback inhibition in vivo are sensitive to the short-term effects of adult thymectomy. B6 mice were thymectomized or sham thymectomized at 6 wk of life, and injected with the indicated cell populations at 10 wk of age. The protocol for adoptive transfer of SRBC-immune Lyl cells and nonimmune T-cells, immunization of adoptive hosts with SRBC and measurement of PFC were performed as outlined in the legend to Fig. 1; three to four mice per group. Similar results were obtained in two additional experiments.

period, NZB mice develop inordinately *high* proportions of Lyl and Ly23 cells, and substantially *reduced* concentrations of Ly123$^+$ cells compared with age and sex-matched BALB/c mice (Fig. 4).

In view of the substantial levels of Ly23$^+$ cells, one explanation of the immunoregulatory defect noted in NZB mice is that the suppressive Ly23$^+$ T-cell set is functionally defective and not capable of generating suppressive activity. This is unlikely since in vitro stimulation of NZB T-cells by high concentrations of SRBC induces potent Ly23$^+$ SRBC-specific suppressive activity, as tested in fresh cultures of NZB spleen cells (10).

An alternative explanation of the immunoregulatory disorder in NZB mice is

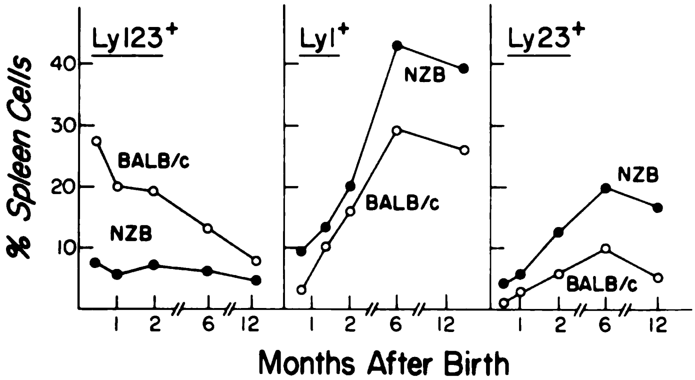


FIG. 4. The ontogeny of Ly$^+$ T-cell sets in NZB and BALB/c mice. The proportions of Ly123$^+$, Ly1$^+$ (Ly1$^+$23$^-$), and Ly23$^+$ (Ly1$^-$23$^+$) T-cell subclasses in the spleen cell populations of (♀) NZB and BALB/c mice of increasing age were measured as described previously (5); specificity controls were performed as indicated in Materials and Methods. Ly phenotypes of spleen cell populations from each age group were determined using pooled spleen cell populations from four to six donors. It should be noted that at 6 mo of age, almost 70% of NZB spleen cells were Ly$^+$ (7% Ly123$^+$; 41% Ly1$^+$; 20% Ly23$^+$). In two additional experiments, spleen cell populations obtained from 6 mo old NZB mice contained 54–70% Ly$^+$ lymphocytes.

that the formation of autoantibodies is, in part, due to high levels of Ly1 T_H activity and the absence of a set of Ly123 cells that normally mediates feedback suppressive activity. To test this hypothesis, spleen cells from NZB mice of different ages were tested for the presence of feedback-suppressive activity in vitro. As early as 1.5 mo after birth, little or no feedback-suppressive activity is detectable in nonimmune NZB spleen cells, in sharp contrast to the potent feedback suppressive activity expressed by spleen cells for age and sex-matched BALB/c controls (Fig. 5). These findings suggest that, both by the criterion of cell surface phenotype and that of in vitro function, NZB mice progressively lose a set of T cells responsible for feedback-suppressive activity.

Discussion

These findings show that T-T interactions responsible for feedback regulatory effects of in vitro antibody responses, also play a central role in regulating in vivo antibody responses. Repopulation of "B" mice with SRBC-immune Ly1 cells resulted in the acquisition of immunocompetence and the formation of large numbers of anti-sheep erythrocyte PFC after immunization. Infusion of unselected, nonimmune T-cells to these mice resulted in a dramatic reduction of the anti-SRBC response. Removal of Ly123$^+$ cells from the nonimmune T-cell population before adoptive transfer eliminated suppression. The set of Ly123 cells responsible for feedback suppression in vivo was shown to be sensitive to small doses of cyclophosphamide and ATx.[2]

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[2] These findings indicate that failure of lymphoid cells from immune donors to transfer secondary antibody responses to adoptive, nonirradiated recipients probably reflects induction of feedback suppressive effects by radiosensitive or cyclophosphamide-sensitive host T cells, rather than a requirement for "space" in host lymphoid tissues. This feedback mechanism should be taken into account in designing clinical protocols aimed at either specific augmentation of immunity by adoptive cellular immunotherapy, or nonspecific augmentation by adjuvant-like materials. Relevant, too, is that the combination -ATx + cyclophosphamide- is synergistic.

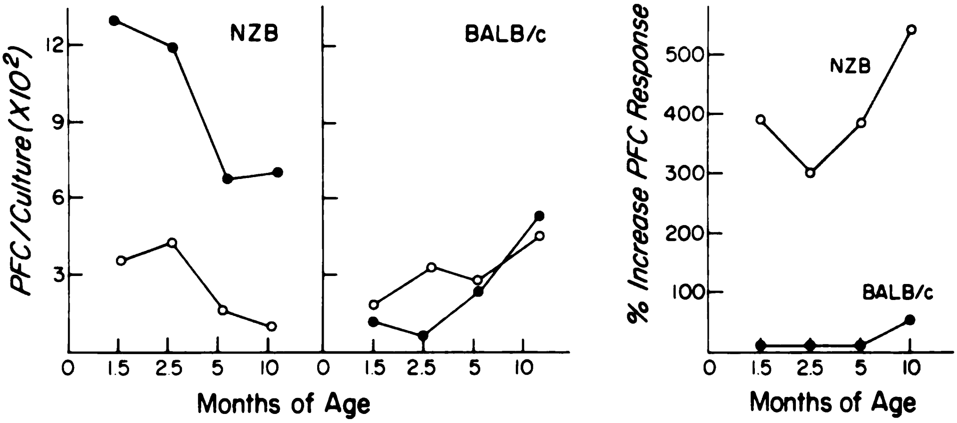

FIG. 5. Failure of spleen cell populations from NZB mice to express feedback inhibition. Purified Ly1 cells were obtained from 10 wk old NZB or BALB/c mice that had been immunized with 2 × 10^8 SRBC i.v. 10 days previously. 2 × 10^5 SRBC-immune Ly1 cells were added to SRBC-stimulated cultures containing 5 × 10^6 syngeneic spleen cells from non-immune NZB or BALB/c mice of the indicated age. The mean α-SRBC-PFC responses of SRBC-stimulated spleen cell cultures from nonimmune NZB or BALB/c mice of increasing age in the absence of added SRBC-immune Ly1 cells (○) are compared to the PFC responses of cultures containing spleen cells combined with 2 × 10^5 syngeneic SRBC-immune Ly1 cells (●). The percent increase in α-SRBC PFC noted after addition of syngeneic SRBC-immune Ly1 cells to age and sex-matched NZB or BALB/c spleen cells is shown on the right.

A second important indication of the physiologic importance of Ly123-mediated feedback suppressive activity comes from studies of NZB mice. These mice (*a*) spontaneously develop a variety of autoantibodies including anti-erythrocyte and anti-DNA antibodies (11), (*b*) acquire cellular and immunologic competence earlier than most other inbred strains (12), perhaps due to accelerated processing of pro-thymocytes by the thymus (13), and (*c*) are difficult to render tolerant after exposure to high concentrations of foreign proteins (14). The present studies clearly establish that throughout the 1st yr of life, spleen cells of NZB mice contain high concentrations (and absolute numbers) of cells of the Ly1 (T_H) set, high levels of Ly23 (T_S) cells, but harbor substantially reduced numbers of Ly123 T-cells. Consonant with this latter finding is the inability of spleen cells from either young or old NZB mice to mediate significant feedback suppressive activity in vitro. These findings, taken together, suggest an association between a "natural" defect in feedback suppression and a loss of immunoregulation resulting in the formation of autoantibodies. These experiments do not establish the cause of this subset-specific T-cell defect. Two possibilities must be considered: (*a*) NZB mice (as well as senescent mice) contain relatively high levels of "naturally-occurring" thymocyte autoantibody (NTA) (15); administration of NTA to young NZB mice can accelerate the disease (15). Possibly this antibody may selectively eliminate the $Ly123^+Qa1^+$ T-cell set responsible for feedback suppression. A second possibility is that NZB mice may undergo chronic, continuous antigen stimulation by virus-associated antigens. Such chronic stimulation might result in accelerated T-cell differentiation and continuous induction of Ly1 and Ly23 T-cell sets, at the expense of a regulatory Ly123 precursor pool. Each of these explanations is clearly testable.[3]

We have not yet established the developmental relationship between the set Ly123$^+$Qa1$^+$ T-cells that mediate feedback suppression and other T-cell sets; nor do we have direct evidence bearing on whether suppression is directly mediated by Ly123 cells, or whether, after stimulation by Ly1 T_H cells, these cells differentiate to "mature" suppressor cells, expressing the Ly23 surface phenotype (17). Tada (18) has indicated that after immunization, products of Ly23 cells can induce immune but not resting Ly123 cells to generate increased antigen-specific Ly23$^+$ T_S activity. A similar recruitment or augmentation circuit has been implied for generation of mature T_H cells: products of Ly1 T_H cells induce Ly123$^+$ T-cells from immune donors to differentiate to mature antigen-specific T_H (Ly1 cells). Both T-T interactions represent *amplification* circuits, in which mature antigen-specific T cells recruit additional T_H or T_S cells from a partially differentiated T-cell population. Both "positive-feedback" circuits may be useful for potentiation of either the helper or suppressive mode after stimulation by antigen. By contrast, the T-T interaction described here represents a "negative-feedback" circuit that is useful for homeostatic regulation of the duration and intensity of the immune response.

These considerations also suggest that the response to a given antigenic determinant may reflect, in part, the amount of feedback inhibition generated after exposure to that antigen. B. Benacerraf et al., personal communication, have recently shown that pretreatment of genetically controlled nonresponder "suppressor" mouse strains with a small dose of cytoxan allows significant PFC responses to two Ir-regulated antigens, GT and GAT. R. K. Gershon has also found that ATx allows nonresponder SJL mice to produce antibody to GAT. These findings indicate that, at least for some Ir gene-controlled antigen responses, exaggerated induction of cytoxan and ATx-sensitive feedback inhibitory cells may be sufficient to mask delivery of the T_H signal to the B cell.

In sum, the data described in this report suggest that the following cellular events may ensue after stimulation of the immune system by foreign materials: activated antigen-specific T_H (Ly1) cells induce B cells to form antibody and induce resting Ly123$^+$Qa1$^+$ cells to inhibit T_H activity (2, 3). Reduction in T_H activity is accompanied by decreased induction of B cells as well as progressively decreasing induction of resting Ly123 cells: the net result is a progressive decrease in both antibody formation and supressor-cell induction.

According to this notion, the following predictions can be made: (*a*) after immunization, T-helper activity should always be accompanied by T-suppressor activity; (*b*) optimal generation of T_S activity requires the presence of both Ly1 cells and Ly123 cells during antigen stimulation; and (*c*) T-helper (Ly1) cells may produce cell-free factors which can induce resting Ly123 cells to express T_S activity. Evidence in support of predictions *a* and *b* have been published (19–21). We have obtained preliminary evidence in support of the third proposition and these latter findings also indicate that T_H (Ly1) cell products that induce B cells to secrete antibody are not biochemically identical to those responsible for the induction of feedback suppression by Ly123 cells.

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[3] It is worth noting that the proportions of Ly123 cells and associated feedback-suppressive activity are substantially reduced in aged mice of all inbred mouse strains so far examined (B6, BALB/c, CBA); possibly the NZB syndrome may, in part, represent a disorder best characterized as accelerated immunologic senescence. Indeed, autoantibodies similar or identical to those found in NZB mice can be found in senescent mice of various other inbred strains (16).

In general, these considerations suggest that the immune system responds in large part to signals continuously generated from *within* the system itself, and that detectable immune responses reflect alterations of these signals after the Ly1 system is perturbed by "antigen". Our data also indicates that a major role of cells of the Ly123 subclass is to (*a*) detect these messages and (*b*) pass them on to mature regulatory T cells of the Ly1 or Ly23 sets (perhaps via macrophages) and/or differentiate to mature regulatory T-cell sets. The net effect of these interactions after perturbation by antigen is to restore the homeostatic balance of the system, usually at a new level reflecting differentiation of antigen-specific clones belonging to the lymphocyte sets described in these studies.

Summary

We have shown that (*a*) purified T-helper cells induce cells of another T-cell set, expressing the $Ly123^+Qa1^+$ surface phenotype, to exert potent suppressive activity, (*b*) this T-T interaction plays an important role in regulating in vivo immune responses, and (*c*) this interaction represents an important barrier to protocols intended to augment the immune status of individuals by adoptive (or active) immunotherapy.

Our results also indicate that the $Ly123^+$ T-cell set mediating feedback suppression in vivo is sensitive to both low doses of cyclophosphamide and removal of the thymus in adult life. The importance of this T-T interaction to normal, physiologic regulation of the immune system is emphasized by the finding that the major T-cell deficit of NZB mice (an inbred strain of mice that spontaneously develops an autoimmune disorder) is the absence or malfunction of an $Ly123^+$ T-cell set responsible for feedback inhibition.

Received for publication 29 December 1978.

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THE EFFECT OF HELPER VIRUS ON ABELSON VIRUS-INDUCED TRANSFORMATION OF LYMPHOID CELLS*

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In the transformation of fibroblasts by defective sarcoma viruses, the role of the helper virus has usually been interpreted as the provision of virion proteins. These may include the internal structural components, the external glycoproteins, and/or the reverse transcriptase (1-4). The ease with which nonproducer, transformed cells can be isolated (5, 6) has suggested that the helper genome plays no role in transformation by defective sarcoma viruses and in certain cases direct examination of transformed cells has confirmed the absence of certain helper nucleic acid sequences (7, 8). Thus, we view fibroblast transformation solely as a consequence of the action of a gene or genes of the defective virus, a view which is supported by the apparent independence of the avian *src* gene from the rest of the nondefective Rous sarcoma virus genome (9, 10). With this background we have been analyzing the helper specificity for transformation of lymphocytes by the Abelson murine leukemia virus (A-MuLV).[1]

A-MuLV, originally isolated from a tumor arising in a prednisolone-treated BALB/c mouse inoculated with Moloney murine leukemia virus (M-MuLV), induces a thymus-independent lymphoma 3-5 wk postinoculation (11). A-MuLV resembles rapidly transforming murine sarcoma viruses in its mode of replication on fibroblast cell lines. The virus stocks from A-MuLV-induced tumors contain a defective virus which transforms some fibroblasts and an oncogenic thymic lymphoma helper virus, M-MuLV. The transforming component of A-MuLV can be obtained free of replicating helper virus by isolating nonproducer fibroblast cell lines (12). Several of these cell lines lack significant sequences of the helper virus genome (7, 8).

A-MuLV is unique in that it also transforms lymphoid cells in vitro (13). A quantitative semisolid culture system which monitors the frequency of transformation has been developed (14). To analyze the role of helper virus in A-MuLV-induced transformation, we tested the oncogenic activity and the transforming

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* Supported by grant no. VC-4I from the American Cancer Society and grant no. CA-14051 from the National Cancer Institute.

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[1] *Abbreviations used in this paper:* A-MuLV, Abelson murine luekemia virus; M-MuLV, Moloney murine leukemia virus; DME-CS, Dulbecco's modified Eagle's medium supplemented with 10% calf serum; DME-IFS, Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated fetal calf serum; E-MuLV, BALB/c endogenous virus WN1802N-C135; F-MuLV, Friend MuLV; G-MuLV, Gross-MuLV; moi, multiplicity of infection; FFU, focus-forming units; PFU, plaque-forming units; PBS, phosphate-buffered saline; RPMI medium, RPMI-1640 supplemented with 20% heat inactivated fetal calf serum and 50 μM 2-mercaptoethanol.

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TABLE I

Origin of Helper Viruses

Virus stock	Abbreviation used
Moloney murine leukemia virus, clone 1 (reference 18)	M-MuLV-1
Moloney murine leukemia virus, clone 2	M-MuLV-2
Friend murine leukemia virus (N-tropic), clone 13	F-MuLV-N
Friend murine leukemia virus (B-tropic), clone 10	F-MuLV-B
BALB/c endogenous virus WN1802N-C135 (references 19 and 20)	E-MuLV
Gross murine leukemia virus	G-MuLV

ability of A-MuLV stocks prepared with several different helper viruses. For fibroblasts, all of the murine retroviruses tested were equivalently effective helpers and A-MuLV stocks prepared with helpers that are highly oncogenic were found to be highly oncogenic in vivo and to transform lymphoid cells efficiently. We were surprised, however, to find that A-MuLV prepared with helpers that are weakly oncogenic alone is very inefficient as a transforming agent either in vivo and in vitro. This result suggests that the helper virus may play a more active role in lymphoid cell transformation than in fibroblast transformation.

Materials and Methods

Cell Cultures. Most fibroblast cell cultures were maintained in Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% calf serum (Microbiological Associates, Walkersville, Md.) (DME-CS). JLS-V9 and CCL64 mink lung cells were maintained in DME supplemented with 10% heat inactivated (at 56°C for 30 min) fetal calf serum (Microbiological Associates) (DME-IFS). 5H cells, an A-MuLV-transformed nonproducer fibroblast clone, were derived by infecting JLS-V9 cells at a multiplicity of infection (moi) of 3 focus-forming units (FFU) and 2 plaque-forming units (PFU) with A-MuLV(M-MuLV-1) (see Table I for a description of the helper viruses used). After a 1.5-h adsorption period, the cells were washed two times with phosphate-buffered saline (PBS) (200 mg/liter KCl, 200 mg/liter KH_2PO_4, 100 mg/liter $MgCl_2 \cdot 6H_2O$, 8,000 mg/liter NaCl, 1,150 mg/liter $Na_2HPO_4 \cdot 2H_2O$), trypsinized for 7 min at 37°C, and plated in microtiter wells at a concentration of 0.33 cells/well. Transformed clones were isolated 14 days later and screened for virus production by using infectivity and reverse transcriptase assays. 5H cells are morphologically transformed but supernates from these cells contain no detectable infectious virus or reverse transcriptase. The cells contain the A-MuLV genomes as demonstrated by rescue experiments with M-MuLV-2.

Lymphoid Cell Lines. A-MuLV-transformed lymphoid cell lines were derived by removing single foci from agarose transformation plates with a Pasteur pipette. The cells were transferred to 16-mm plastic wells (Corning Glass Works, Corning, N. Y.), suspended in 1 ml of RPMI-1640 (Grand Island Biological Co.), supplemented with 20% heat inactivated fetal calf serum and 50 μM 2-mercaptoethanol (Eastman Kodak Co., Rochester, N. Y.) (RPMI medium). Cultures were observed daily for growth and maintained at densities of 5×10^5–1×10^5 cells/ml until the cultures grew vigorously, doubling every 12 h. The length of this adaptation period usually varied from 3 to 4 days to 1–2 wk. Once established, cells were passaged at a 1:100 split ratio when they reached a density of 2.5–3.5×10^6 cells/ml, about every 3rd day.

BR48 is an A-MuLV-transformed lymphoid nonproducer cell line derived from A-MuLV(M-MuLV-1)-infected C57BR/cdJ (Fv-$1^{n/n}$) bone marrow cells. 2M is another A-MuLV-transformed nonproducer cell line derived from A-MuLV(M-MuLV-2) infected BALB/c (Fv-$1^{b/b}$) bone marrow cells. Both of these cell lines contain the A-MuLV genome as demonstrated by recovery of oncogenic, transforming virus after M-MuLV-2 superinfection.

L691 cells, a gift of Dr. Paul Arnstein, California State Department of Health, were derived from a radiation-induced thymoma in a C57L mouse (15). These cells express markers commonly associated with T-cell tumors such as Thy-1, TL, and Lyt-4 antigens, and the enzyme terminal deoxynucleotidyl transferase (16, 17; A. Silverstone et al., unpublished results). No infectious ecotropic virus has been isolated from the cells, but xenotropic virus can be recovered by cocultivation of L691 cells with CCL64 mink lung cells or other non-murine cell lines (15; N. Rosenberg and D. Baltimore, unpublished results).

Virus Stocks. M-MuLV-1, M-MuLV-2, Friend-MuLV(F-MuLV)-N, Gross-MuLV(G-MuLV), G-MuLV-1, and E-MuLV were all grown as NIH/3T3 carrier cell cultures (Table I). F-MuLV-B was maintained as a BALB/3T3 carrier culture. M-MuLV-1 was originally isolated from JLS-V11 cells by end point dilution as previously described (18). M-MuLV-2 was isolated from M-MuLV-1 by cloning NIH/3T3 cells after adsorption of virus at low multiplicity. E-MuLV was isolated by end point dilution of WN1802N (19) as previously described (20). G-MuLV, originally obtained from Dr. Nancy Hopkins, M.I.T., was cloned by infecting NIH/3T3 cells at a moi of 0.3 PFU. After a 1.5-h adsorption period, the infected cells were trypsinized and plated in microtiter plates at a concentration of 0.3 cells/well. 10 days later, all wells containing cells were assayed for virus production by the XC plaque assay (21). 2 of 51 clones released infectious virus, and one of these, G-MuLV-1, was selected for use in experiments. Cloned F-MuLV stocks originally obtained from Dr. Robert Eckner, Boston University School of Medicine, Boston, Mass. were a gift of Dr. Alfred Bothwell, M.I.T. F-MuLV-N and F-MuLV-B were isolated by low moi and cloning of SC-1 cells. C57Bl/KaB (22), a clone of fibrotropic C57Bl/6 virus, was a gift of Doctors Henry Kaplan and Alain Declève, Stanford University, Stanford, Calif.

A-MuLV stocks were prepared by superinfecting mass cultures of nonproducer fibroblast cells with helper virus. After a 2-h adsorption period, the cells were maintained at high densities with frequent medium changes for 1.5-2 wk to allow virus spread. Both helper virus and A-MuLV stocks were prepared from 24-h harvests of exponentially growing cultures. The virus supernates were centrifuged for 15 min at low speed, filtered through a 0.45-μ filter (Millipore Corp., Bedford, Mass.), and frozen at −70°C for subsequent use.

For quantitative rescue experiments with lymphoid nonproducer cells, 2×10^6 cells were resuspended directly in 1 ml of the helper virus preparations supplemented with 8 μg/ml polybrene (Aldrich Chemical Co., Milwaukee, Wis.). After a 1.5-h adsorption period, the cells were collected by low speed centrifugation, washed once with 10 ml of PBS, and treated with 0.25% trypsin, 0.2% EDTA for 7-10 min at 37°C. Trypsin-EDTA was removed by low speed centrifugation and the cells were plated in 4 ml of RPMI medium. Subsequently, culture fluids were collected for virus assay at 24-h intervals. The cells were collected and replated at the initial cell density (5×10^5 cells/ml) in fresh medium for the next collection interval.

Virus Assays. Helper viruses were assayed on NIH/3T3 and BALB/3T3 cells by using the standard XC assay or by mouse S+L- assay with FG-10 cells (23). FG-10 assays were carried out by plating 1×10^5 cells in 60-mm plastic dishes. 24 h later, the cells were infected with 0.5 ml of virus. After a 2-h adsorption period in the presence of 8 μg/ml polybrene, the cells were fed 4 ml of DME-CS and incubated. The S+L- plaques were visualized by hematoxylin staining 5 days postinfection. The titers obtained with the XC and FG-10 assays were equivalent for all the virus stocks (data not shown).

Fibroblast transformation assays were used to standardize the FFU in A-MuLV stocks. Either 1×10^5 NIH/3T3 or BALB/3T3 cells, plated in 60-mm dishes 24 h previously, were infected with 0.5-ml portions of virus. After a 2-h adsorption period in the presence of 8 μg/ml polybrene, the plates were fed fresh DME-CS, and incubated. Medium was changed 5 and 10 days postinfection and foci were scored in live cultures by using low power magnification (× 100) at 15 days postinfection (12). The amount of helper virus present in A-MuLV stocks was determined by using SC or S+L- assays.

All helper virus and A-MuLV preparations were screened for the ability to replicate on xenogeneic cells. For this assay, CCL64 mink lung cells, plated 24 h previously at 1×10^5 cells/60 mm dish, were infected with 0.5 ml of undiluted virus stock containing 8 μg/ml polybrene. After a 2-h adsorption period, the cells were fed fresh DME-IFS and incubated. Cells were passaged twice weekly and screened for the presence of virus by using a standard reverse transcriptase assay 6 wk postinfection.

Bone Marrow Transformation. Transformation of mouse bone marrow cells was carried out as previously described (14). Briefly, 2×10^6 nucleated cells were plated in 35-mm dishes in 1 ml of RPMI medium. 1 ml of virus suspension was added to the dishes and the plates were incubated. $^1/_2$ ml of fresh RPMI medium was added to the cultures 6 and 12 days postinfection. Transformation was recognized by rapid proliferation of immature lymphoid cells in the culture fluid.

Mice. The mice used for most experiments were supplied by our breeding colony at M.I.T. NIH/Swiss mice were originally obtained from the Division of Research Services, National Institutes of Health, Bethesda, Md. BALB/cAN mice were obtained from Dr. Herman Eisen, M.I.T. CD-1/Swiss mice were purchased from Charles River Breeding Laboratories, Wilmington, Mass. For certain experiments, C57BR/cdJ mice were purchased from The Jackson Laboratory, Bar Harbor, Maine.

For induction of leukemia all animals were inoculated intraperitoneally with 0.1 ml of 0.45 μm of filtered virus suspension at less than 48 h of age. Animals were observed weekly for cachexia, anemia, lymphadenopathy, or splenomegaly. Mice inoculated with M-MuLV clones and F-MuLV clones remaining disease free were sacrificed 6 mo postinoculation. Animals inoculated with E-MuLV and G-MuLV were held for 15 mo. Mice inoculated with all A-MuLV stocks were observed for 3 mo. Gross pathologic findings were noted for all animals either when illness was noted or at the termination of the experiments. Spleen, thymus, lymph node, liver, and any other abnormal tissues from representative animals were also fixed and processed for histologic examination according to routine procedures.

For studies on recovery of A-MuLV from mice, several NIH/Swiss litters were randomly mixed and then redivided for virus inoculation. Each mouse received equivalent doses of either A-MuLV(M-MuLV-1) or A-MuLV(G-MuLV). Animals were sacrificed by cervical fracture 4, 10, 15, and 21 days postinfection (and, in the case of A-MuLV(M-MuLV-1), when disease was evident). The animals were examined for evidence of disease and spleen, thymus, femur, and tibia were removed and frozen immediately on dry ice. Virus extracts, prepared in RPMI containing no serum, were made by using a dounce homogenizer or a mortar and pestle. The extracts were centrifuged twice at 2,500 rpm for 30 min and filtered through a 0.45-μm Millipore filter before infectivity studies. Protein concentration in the extracts was determined by the standard Lowry et al. procedure (24).

Reverse Transcriptase Assays. 24-h supernates from exponentially growing cell cultures were clarified by low speed centrifugation. The supernates were then centrifuged at 100,000 *g* for 45 min at 4°C. Pellets were resuspended in 0.5 ml of 0.01 M Tris-HCl (pH 7.5) and sonicated for 40 s in a Raytheon sonic oscillator, Raytheon Co., Portsmouth, R.I. A 50-μl portion of the virus suspension was assayed for exogenous reverse transcriptase activity in 0.1 ml reaction mixture containing 0.05 M Tris-HCl buffer (pH 8.3), 0.06 M NaCl, 6 mM $MnCl_2$, 0.02 M dithiothreitol, 1 μg poly(rA) (Miles Laboratories, Inc., Kankakee, Ill.), 0.5 μg oligo(dT) (Collaborative Research, Inc., Waltham, Mass.), 0.05% Nonidet P-40 (Gallard Schlesinger Chemical Mfg. Corp., Carle Place, N.Y.), and 10^{-5} M [^{3}H]dTTP (52 Ci/mmol) (New England Nuclear, Boston, Mass.).

Results

The tropism of the helper viruses described in Table I was determined by using the XC plaque assay method (21) on NIH/3T3 and BALB/3T3 cells (Table II). Both cloned stocks of M-MuLV produced equivalent numbers of large, clear plaques on the two cell lines and were therefore NB-tropic (25). The two F-MuLVs were of opposite tropism, one preferring NIH/3T3 cells (F-MuLV-N) and the other BALB/3T3 cells (F-MuLV-B). The G-MuLV stocks and E-MuLV clone were N-tropic, producing small, clear XC plaques preferentially on NIH/3T3 cells. All of the viruses replicated to high titer in fibroblast cells of the appropriate Fv-1 genotype. None of the virus stocks had an amphotropic host range as determined by their inability to establish an infection in mink cells.

The oncogenicity of helper virus preparations was determined by inoculating newborn mice of proper Fv-1 genotype with 0.45 μm filtered virus stocks (Table III).[2] Mice were sacrificed when signs of disease were evident and gross and histologic examinations were conducted. Both M-MuLV-1 and M-MuLV-2 induced disease in 96–100% of both Swiss and BALB/c mice after a 2.5–3.5-mo latent period. Hepatosplenomegaly and large mesenteric lymph node tumors

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[2] Jolicoeur et al. *J. Natl. Cancer Inst.* In press.

TABLE II
Properties of Helper Viruses

Virus stock	Titer		Growth on mink lung cells
	NIH	BALB	
M-MuLV-1	2×10^6	1×10^6	–
M-MuLV-2	2×10^6	1×10^6	–
F-MuLV-N	4×10^5	1×10^2	–
F-MuLV-B	<10	1×10^6	–
E-MuLV	1×10^4	1×10^2	–
G-MuLV	3×10^5	1×10^2	–
G-MuLV-1	2×10^5	8×10^2	

0.5-ml portions of filtered virus dilutions were exposed to NIH/3T3 or BALB/3T3 cells plated 24 h earlier at densities of 5×10^4 cells or 7.5×10^4 cells/60 mm dish. After a 2-h virus adsorption period, cells were fed fresh DME-CS and incubated. 3 days later the plates were irradiated with ultraviolet light and 1×10^6 XC cells were added. Plaques were visualized by hematoxylin staining.

Growth of virus stocks on mink lung cells was monitored by reverse transcriptase assay 6 wk postinfection.

were prominent features of the disease and thymomas were noted in about 50% of the animals. Histologic examination of tumor tissue showed that the tumors were composed of lymphoblasts. M-MuLV-2 titrations in NIH/Swiss mice indicated that doses less than 6×10^3 were effective in disease induction (data not shown).

F-MuLV-B and F-MuLV-N were also oncogenic. Both viruses induced diseases confined to the spleen and liver. Mesenteric and peripheral lymph nodes and the thymus of inoculated mice were normal. Histologic examination of the diseased tissues showed the F-MuLV-B-induced tumors to be composed of lymphoblasts, morphologically indistinguishable from the cells in M-MuLV-induced tumors. As with M-MuLV, F-MuLV-B induced a disease after a 2.5–3.5-mo latent period. F-MuLV-N induced disease after a short, 5–6 wk, latent period. The types of cells in F-MuLV-N-induced tumors have not been fully characterized. The virus stocks did induce disease in adult mice but did not induce spleen foci characteristic of Friend virus complex with the standard 9 day focus assay (26) (data not shown).

Neither E-MuLV nor G-MuLV induced tumors in NIH/Swiss mice. Inoculated animals were observed for 15 mo and no gross or histologic evidence of tumors was noted.

Characterization of Abelson Stocks. Two A-MuLV-transformed nonproducer fibroblast cell lines were used to prepare A-MuLV stocks with the various helpers. ANN-1 cells (12), derived from NIH/3T3 cells, were used to prepare virus stocks with N- and NB-tropic helpers and 5H cells, derived from BALB/c JLS-V9 cells (27), were used to prepare virus stocks with B- and NB-tropic helpers. The focus-forming component of all virus stocks was monitored by transformation of NIH/3T3 or BALB/3T3 cells and the titers are represented as FFU. Helper virus titers were determined by using XC or S+L- plaque assays and are represented as PFU. All A-MuLV stocks transformed fibroblasts of

TABLE III
Oncogenicity of Helper Viruses

Virus stock	Titer inoculated (PFU)	Mouse strain	Disease frequency	Latent period	
				Mean	Range
				days	
M-MuLV-1	1×10^4	N	15/15	102	78–118
	1×10^4	B	30/31	83	77-91
M-MuLV-2	7×10^3	N	6/6	92	72-109
	3×10^5	B	6/6	109	94-128
F-MuLV-N	1×10^4	N	5/6	37	34-39
E-MuLV	3×10^4	N	0/11	–	–
G-MuLV	3×10^4	N	0/11	–	–

Neonatal mice were inoculated intraperitoneally with filtered virus stocks and observed for signs of disease. Animals inoculated with E-MuLV and G-MuLV were observed for 15 mo. The mouse strains used were either CD-1 or NIH/Swiss (N) and BALB/c (B). CD-1 and NIH/Swiss mice are equally susceptible to these lymphoma viruses.

appropriate Fv-1 genotype efficiently and reflected the tropism of the helper virus (Table IV). The proportion of helper virus in virus stocks varied with the preparation but most of the virus stocks had PFU/FFU ratios of 1-5. Virus stocks prepared with M-MuLV-1 and ANN-1 cells and one ANN-1-grown F-MuLV-N stock contained virus that could replicate in mink lung cells. Occasionally, preparations made with 5H cells contained virus that could replicate on mink cells after long-term infection (Table IV). Xenotropic virus probably arises as a low level contaminant from the rescued A-MuLV nonproducer cell lines. Most transformed lymphoid cell lines isolated with these virus stocks do not release virus which grows on mink lung cells (data not shown).

To determine the relationship between FFU and Abelson disease induction, an in vivo A-MuLV titration was performed initially by using A-MuLV(M-MuLV-2) and NIH/Swiss mice. Mice were sacrificed when cachexia, lymphadenopathy, paralysis of hind limbs, or meningeal tumors evident grossly as a bulging of cranial bones were noted. Animals with lymphosarcomas involving bone marrow and peripheral lymph nodes with slight splenomegaly and normal thymus and mesenteric lymph nodes were scored as positive for Abelson disease. An inoculum of 10^3 FFU induced disease in more than 50% of the test animals while only 1 of 11 mice inoculated with 5×10^2 FFU developed Abelson disease (Table V).

A-MuLV prepared with the various helper viruses was then inoculated into newborn mice at doses expected to give a high disease frequency based on the A-MuLV(M-MuLV-2) titration. A-MuLV stocks prepared with both clones of M-MuLV induced disease efficiently in NIH/Swiss and BALB/c mice after a 3–5-wk latent period (Table VI). Typical Abelson lymphosarcomas, characterized by large tumors of peripheral lymph nodes, spine, and skull with slight splenomegaly and normal thymus, were found in these animals. Histologically, the tumors were composed of immature lymphoid cells. Disease after A-MuLV(F-MuLV-B) inoculation was similar to the syndrome observed when A-MuLV(M-MuLV) clones were used. The A-MuLV(F-MuLV-B) stocks were more efficient in BALB/c than NIH/Swiss mice.

TABLE IV
Properties of A-MuLV Stocks

Virus stock	PFU Titer		FFU Titer		Growth on mink lung cells
	NIH	BALB	NIH	BALB	
ANN-1(M-MuLV-1)	1×10^6	7×10^5	1×10^6	7×10^5	+
ANN-1(M-MuLV-2)	1×10^6	ND*	5×10^5	5×10^5	ND
ANN-1(F-MuLV-N)	2×10^5	4×10^1	7×10^5	<10	+/−
ANN-1(E-MuLV)	2×10^4	3×10^2	4×10^4	1×10^2	−
ANN-1(G-MuLV)	8×10^4	3×10^2	1×10^5	8×10^1	−
5H(M-MuLV-2)	3×10^5	2×10^5	9×10^4	4×10^4	+
5H(F-MuLV-B)	<10	1×10^5	<10	2×10^5	+/−

0.5-ml portions of filtered virus dilutions were exposed to NIH/3T3 or BALB/3T3 cells for assay by the standard XC plaque test or the A-MuLV fibroblast focus assay. For the XC test, XC cells were added to monolayers irradiated with ultraviolet light 3 days postinfection and plaques were scored 3 days later. A-MuLV transformed foci were scored 15 days postinfection. Growth of virus on mink lung cells was monitored by reverse transcriptase assay 6 wk postinfection. The A-MuLV stocks are described by the nonproducer cell from which they were derived and, in parenthesis, the helper virus used for rescue.

* ND, not done.

NIH/Swiss mice inoculated with A-MuLV(F-MuLV-N) also developed typical Abelson disease (Table VI). However, in all of the mice inoculated with this virus stock, hepatosplenomegaly was a prominent feature of the disease. This type of pathology is very uncommon in Abelson disease and probably reflects the action of the F-MuLV-N helper virus in the preparation. The overlapping latent period for F-MuLV-N disease and Abelson disease makes it difficult to assess which cells have responded to which virus. Skull, spine, and peripheral lymph node tumors, characteristic of Abelson disease, and never noted in F-MuLV-N injected mice, were present in about 40% of the animals. The high titer of helper virus in the particular A-MuLV(F-MuLV-N) preparation used in these animal experiments may account for the high proportion of animals with hepatosplenomegaly.

Mice inoculated with A-MuLV(G-MuLV) did not develop any detectable disease during a 3–4-mo observation period. To determine if A-MuLV(G-MuLV) replicates in inoculated animals, lymphoid tissues from NIH/Swiss mice injected with this virus stock were extracted and examined for virus by using the S+L- assay and the NIH/3T3 focus assay (Fig. 1). Tissues from mice inoculated with an equivalent amount of A-MuLV(M-MuLV-1) served as controls. The virus dose used, 2×10^4 FFU, induced disease in 100% of the A-MuLV(M-MuLV-1) inoculated mice. A-MuLV(M-MuLV-1) replicated well in the mice and as early as 4 days postinjection both components of A-MuLV could be detected in bones of the animals. Helper virus was also recovered from spleen and thymus. As the incubation period increased, the titer of A-MuLV increased. The higher titer of A-MuLV in thymus tissue observed in the tumored mice probably arises from large parathymic lymph node tumors in these animals. No virus was recovered from bone, spleen, or thymus of A-MuLV(G-MuLV) injected mice. Litter mates of the A-MuLV(G-MuLV) mice sacrificed in these experiments are healthy 5 mo postinoculation.

TABLE V
Oncogenicity of A-MuLV(M-MuLV-2)

Titer inoculated		Disease frequency	Latent period	
PFU	FFU		Mean	Range
			days	
1.5×10^5	5×10^4	8/8	24	21-28
1.5×10^4	5×10^3	6/7	33	29-40
3×10^3	1×10^3	3/5	75	71-78
1.5×10^3	5×10^2	1/11	–	78

Neonatal mice were inoculated intraperitoneally with 0.1 ml of filtered virus stock. Animals were sacrificed when disease was evident. Animals were observed for at least 5 mo.

TABLE VI
Oncogenicity of A-MuLV Stocks

Virus stocks	Titer inoculated		Mouse strain	Disease frequency	Latent period	
	PFU	FFU			Mean	Range
					days	
ANN-1(M-MuLV-1)	1×10^5	2×10^4	N	4/4	30	28-30
	ND[*]	2×10^4	B	7/7	19	18-20
ANN-1(M-MuLV-2)	1×10^4	5×10^3	N	8/8	37	29-43
			B	ND	–	–
ANN-1(F-MuLV-N)	2×10^4	4×10^3	N	11/11	41	38-55
			B	ND	–	–
5H(M-MuLV-2)	3×10^4	6×10^3	N	5/9	54	28-87
			B	5/5	28	23-30
5H(F-MuLV-B)	1×10^4	3×10^4	N	1/6	30	30
			B	5/6	31	24-47
ANN-1(G-MuLV)	8×10^3	1×10^4	N	0/14	–	–
			B	ND	–	–

Neonatal mice were inoculated intraperitoneally with 0.1 ml of filtered virus stock. Animals were sacrificed when disease was evident. Animals were observed for at least 3 mo. Mouse strains used were NIH/Swiss (N) and BALB/c (B). The A-MuLV stocks are described by the nonproducer cell from which they were derived and, in parentheses, the helper virus used for rescue.

[*] ND, not done.

Transformation of Bone Marrow Cells. To determine the effect of helper viruses on the ability of A-MuLV stocks to transform hematopoietic cells in vitro, bone marrow cells from 4- to 6-wk old mice were infected and plated in a quantitative agarose transformation assay. Macroscopic foci of lymphoid cells were counted 12 days postinfection (Table VII). The transformation frequency obtained with different virus preparations was standardized by the fibroblast FFU in the virus stocks. A-MuLV in combination with most of the helper viruses efficiently transformed lymphoid cells. Colonies arising in these cultures could routinely be established as continuous cell lines. Plating efficiency in most cases was 80–90% (data not shown).

Cultures infected with A-MuLV(E-MuLV) and A-MuLV(G-MuLV) had very few macroscopic foci (Table VII). In more than 200 A-MuLV(E-MuLV) infected

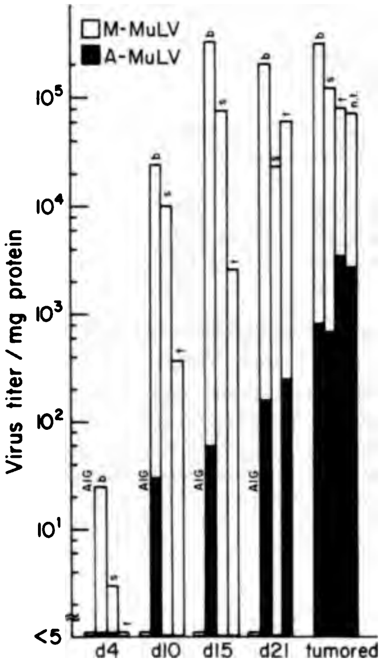

FIG. 1. Recovery of A-MuLV from mice. Extracts of bone (b), spleen (s), and thymus (t) from mice injected with A-MuLV(M-MuLV-1) or A-MuLV(G-MuLV) were assayed for both components of A-MuLV in vitro. Lymph node tumors (n.t.) from terminal animals injected with A-MuLV(M-MuLV-1) were also examined. ■ (A-MuLV) indicates FFU and ☐ (M-MuLV) indicates PFU recovered in the various tissues from A-MuLV(M-MuLV-1)-injected mice. For mice injected with A-MuLV(G-MuLV), results are shown in one column (A/G) although all three tissues were assayed separately.

cultures, three foci were observed. None of these grew independently in liquid culture. One macroscopic focus has been noted in a culture infected with A-MuLV(G-MuLV). The cells in this focus did grow independently and were established as a cell line. The cells appeared to be similar to other A-MuLV transformed lymphoid cells with respect to growth and morphologic properties. The cells did not produce infectious virus, but did contain the A-MuLV genome which could be rescued by superinfection with M-MuLV (data not shown).

To further investigate the transformation potential of A-MuLV stocks, both A-MuLV(E-MuLV) and A-MuLV(G-MuLV) were used to infect mass cultures of bone marrow cells. In this transformation test, virus is mixed with bone marrow cells in liquid culture and the cells are incubated in the presence of input virus and any virus arising by subsequent rounds of replication. Under conditions where an equivalent dose of A-MuLV(M-MuLV-1) transformed 95–100% of the cultures, no transformation was observed in cultures infected with either A-MuLV(E-MuLV) or A-MuLV(G-MuLV).

The ratio of helper virus to fibroblast focus-forming virus in different A-MuLV stocks varies. However, the amount of helper virus in A-MuLV stocks does not appear to influence the transformation potential of the virus (Table VI). Addition of a competent helper virus such as M-MuLV-1 to A-MuLV(G-

TABLE VII

A-MuLV-Induced Transformation of Bone Marrow Cells

Virus stock	PFU/FFU	Lymphoid foci/10^5 fibroblast FFU		Lymphoid foci NIH/Swiss marrow/Lymphoid foci BALB/c marrow	Oncogenic in vivo
		NIH/Swiss	BALB/c		
ANN-1(M-MuLV-1)	6	92	48	1.7	Yes
ANN-1(M-MuLV-2)	5	28	26	1.0	Yes
ANN-1(F-MuLV-N)	3	20	0.25	200	Yes
5H(M-MuLV-2)	3	148	88	1.6	Yes
5H(F-MuLV-B)	0.5	8	103	0.006	Yes
ANN-1(G-MuLV)	0.7	<0.01	ND[*]		No
ANN-1(E-MuLV)	1.5	<0.02	ND		ND

NIH/Swiss or BALB/c mouse bone marrow was infected with A-MuLV in vitro and plated in the standard lymphoid cell transformation assay. Foci were scored 12 days postinfection. The A-MuLV stocks are described by the nonproducer cell from which they were derived and, in parenthesis, the helper virus used for rescue.

[*] ND, not done.

MuLV) virus at the time of infection did not overcome its inability in transformation (data not shown).

As has been observed for fibroblast cells, N-tropic A-MuLV stocks preferentially transform lymphoid cells from Fv-$1^{n/n}$ mice while B-tropic A-MuLV stocks are much more efficient on bone marrow cells from Fv-$1^{b/b}$ mice (Table VII). NB-tropic A-MuLV stocks transform bone marrow cells from both types of mice at high efficiency.

Results obtained in a different experimental system (28) have suggested that Fv-1-mediated restriction does not operate in lymphoid cells. The low transformation frequencies obtained with N- and B-tropic A-MuLV stocks on bone marrow cells of inappropriate Fv-1 genotype suggest that lymphoid cell transformation is subject to the effect of the Fv-1 gene.

Rescue of A-MuLV from Nonproducer Cells. Lymphoid nonproducer cell lines were isolated by removing single foci from agarose culture plates and adapting the cells to growth in liquid medium. Nonproducer cell lines were identified by the lack of reverse transcriptase-containing virus particles in the culture fluid. Two nonproducer cell cultures, BR48 and 2M, were readily superinfected with M-MuLV-2 (Table VIII). After addition of the helper virus, infection and release of both helper and focus-forming virus occurred within 12–15 h. Superinfected cultures could then be maintained as stable virus-producing cell lines.

Initial attempts to make virus-producing cultures of BR48 cells by using G-MuLV as a helper were unsuccessful (Table VIII). A standard fibroblast nonproducer rescue protocol was employed, growing the superinfected cells for 1.5–2 wk before assaying virus. More careful study revealed that G-MuLV did not form a stable association with lymphoid nonproducer cells. After superinfection with G-MuLV, virus production of both helper and focus-forming virus increased for the first 48-h and then declined (Fig. 2). In cultures superinfected with M-MuLV-2, virus production continued in a stable fashion. After virus

TABLE VIII

Superinfection of A-MuLV Transformed Nonproducer Cells

Nonproducer	Helper virus	Virus Replication	
		PFU	FFU
BR48(Fv-$1^{n/n}$)	M-MuLV-2	1×10^5	8×10^4
	F-MuLV-N	1×10^3	2×10^2
	G-MuLV-1	9	2
	E-MuLV	0	0
2M(Fv-$1^{b/b}$)	M-MuLV-2	2×10^5	7×10^4
	F-MuLV-B	1.5×10^6	5×10^5
	C57Bl/KaB	0	ND*

Lymphoid nonproducer cell lines were infected with helper viruses at an moi of 0.5. After a 2-h adsorption period, cells were maintained as exponentially growing cultures for 10–14 days at which time fresh culture fluids were examined for the presence of PFU and FFU. Viruses produced by 2M cells were assayed on BALB/3T3 cells. Viruses produced by BR48 cells were assayed on NIH/3T3 cells.

* ND, not done.

production had ceased in BR48(G-MuLV) cultures, the population could be reinfected with G-MuLV or M-MuLV-2 suggesting that the culture has lost some if not all virus expression from the initial superinfection.

BR48 cells, an Fv-$1^{n/n}$ cell line, could be stably superinfected by F-MuLV-N but not by E-MuLV (N-tropic). 2M, an Fv-$1^{b/b}$ cell line, could be easily superinfected by both M-MuLV-2 and F-MuLV-B but not by the B-tropic C57Bl/KaB virus (Table VIII). G-MuLV, however, did infect a T-cell lymphoma cell line, L691-6, and replicated nearly as efficiently as M-MuLV-2 in these cells (Fig. 3).

Discussion

These studies have shown that A-MuLV oncogenesis and lymphoid cell transformation is dependent on which helper virus rescued and accompanies the defective A-MuLV. Three MuLVs that readily caused lymphomas on their own acted as efficient helpers to produce Abelson disease in vivo and lymphoid cell transformation in vitro. Two MuLVs that induced no detectable disease by themselves also failed to act as efficient helpers for lymphoid cell transformation in vitro and the one that was tested, A-MuLV(G-MuLV), was also inactive in vivo. Neither component of the A-MuLV(G-MuLV) stocks established an infection in the inoculated mice. For fibroblast transformation, however, all five helpers were equally active when normalized to their PFU content. There is, therefore, an important difference between the requirements for A-MuLV transformation of lymphoid cells and of fibroblasts. Scher (29) has observed a similar distinction in studies with Kirsten sarcoma virus and A-MuLV.

Transformation of fibroblasts by A-MuLV is similar to transformation of fibroblasts by murine sarcoma viruses in that any MuLV that can replicate on its own will act as an effective helper (1, 2, 5, 6, 30). What then is the significance of the helper specificity for lymphoid cell transformation and oncogenesis by A-MuLV?

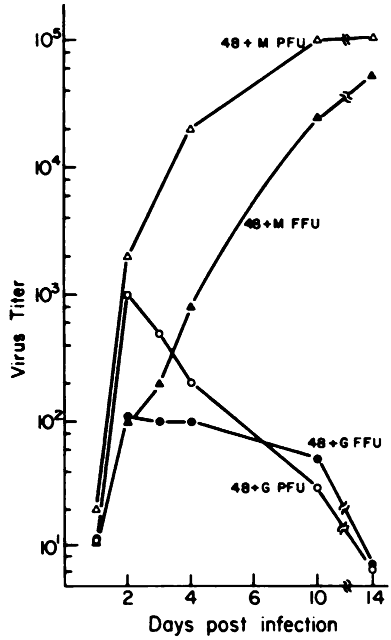

FIG. 2. Replication of virus in A-MuLV-transformed lymphoid nonproducer cells. BR48 cells were infected with M-MuLV and G-MuLV at an moi of 0.1. Virus in the culture fluids was monitored at intervals postinfection by using standard assays.

Two hypotheses are possible. One is that the helper is needed for effective penetration of the A-MuLV genome and that lymphoid target cells have receptors that distinguish the competent helpers from the incompetent ones. The other hypothesis is that the helper plays an intracellular role in initiating or maintaining the transformation.

The penetration hypothesis is supported by the inability of an added competent helper virus to elicit lymphoid transformation by an incompetent A-MuLV stock. Other explanations of this result, however, are possible and further experiments in this area are needed.

As a test of the penetration hypothesis, we examined the ability of A-MuLV transformed nonproducer lymphoid cells to be infected by G-MuLV and M-MuLV. Both viruses established infections in the A-MuLV-transformed cells therefore, it can be argued, that at least after transformation the cells have receptors for both viruses. We cannot test the cells before infection because we cannot make a pure culture of target cells. A further argument against the penetration hypothesis is the ability of G-MuLV and M-MuLV pseudotypes with vesicular stomatitis virus to infect the A-MuLV-transformed nonproducer cells with equal efficiency (P. Besmer et al., unpublished results).

The possibility that the "helper" may play a critical, on-going role in the transformation process receives some support from our studies of particle production by the A-MuLV-transformed lymphoid cell lines. Some of these lines are totally nonproductive but a majority do produce a noninfectious particle (A. Shields et al., unpublished results). This particle has a major internal protein closely related to that of M-MuLV suggesting that the M-MuLV genome may be

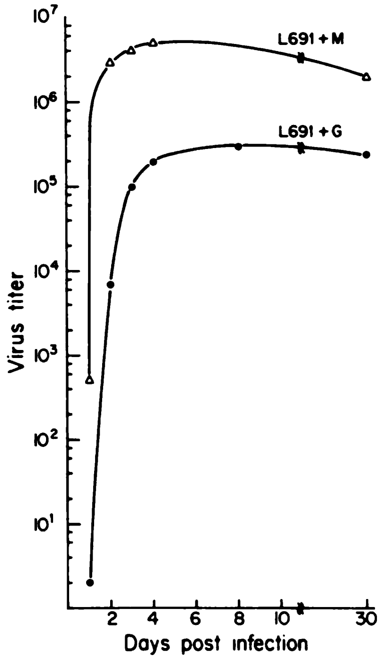

FIG. 3. Replication of helper virus in L691-6 thymoma cells. L691-6 cells were infected with M-MuLV-1 and G-MuLV at an moi of 0.1. Virus in culture fluids was monitored at intervals postinfection by using standard assays.

present in most of the lymphoid transformants. Nucleic acid hybridization experiments will be able to assess whether all transformants might contain some or all of the M-MuLV genome. If the genome is present, it could contribute to maintaining the transformed state. This possibility is not without precedent: certain murine sarcoma viruses only transform in the presence of a helper virus (6, 23).

The transitory production of virus after G-MuLV infection of the A-MuLV-transformed nonproducer cells (Fig. 2) is not understood. The loss of infectivity is so rapid that overgrowth of initially uninfected cells is an unlikely explanation. It seems that G-MuLV, although it can initiate infection, cannot stably maintain the infected state and whether the G-MuLV provirus can even integrate is not known. The inability of G-MuLV to stably infect lymphoid nonproducer cells does not reflect an inability of this virus to infect all lymphoid cells, however, because this virus replicates nearly as well as M-MuLV-2 in L691 lymphoma cells. This inability of G-MuLV to maintain a productive infection of the A-MuLV-transformed cells may be an explanation of its inability to act as an effective helper for A-MuLV transformation.

The inability of E-MuLV, another inefficient helper, to stably infect lymphoid nonproducer cells supports a correlation between a virus' inability to help A-MuLV transform lymphoid cells and its inability to stably associate with lymphoid nonproducer cells. Although the helper efficiency of C57Bl/KaB virus has not been determined, the virus is fibrotropic and nononcogenic in C57Bl/Ka mice (22). Thus, the inability of this virus to stably infect 2M cells probably also fits this pattern.

The correlation between the oncogenicity of a replication-competent MuLV and its ability to act as an efficient helper is striking. This could suggest that the helper virus is providing a critical leukemogenic function in the transformation process. Thus, the data might be reinterpreted by saying that A-MuLV is acting as a helper for the replication competent MuLV in producing the Abelson disease. The Abelson virus would then function to alter the cellular specificity of the leukemogenic virus, e.g. M-MuLV, and allow it to transform more immature lymphoid cells than those usually affected by that virus (17).

These speculations may seem premature but they serve to focus attention on the difference between lymphoid cell transformation and fibroblast cell transformation. Fibroblast transformation may be a relatively simple process whereby a single gene product establishes control of cellular growth but at present leukemia virus transformation has properties that suggest more complex interactions.

Summary

Abelson murine leukemia virus (A-MuLV)-transformed fibroblast nonproducer cells were used to prepare A-MuLV stocks containing a number of different helper viruses. The oncogenicity of the A-MuLV stocks was tested by animal inoculation and their ability to transform normal mouse bone marrow cells was measured in vitro. All of the A-MuLV stocks transformed fibroblast cells efficiently. However, only A-MuLV stocks prepared with helper viruses that are highly oncogenic were efficient in vivo and in vitro in hematopoietic cell transformation. In addition, inefficient helpers did not establish a stable infection in lymphoid nonproducer cells. Thus, helper virus has a more central role in lymphoid cell transformation than in fibroblast cell transformation.

We thank Daniel Clark for excellent technical assistance.

Received for publication 9 November 1977.

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RESTRICTED HELPER FUNCTION OF F_1 HYBRID T CELLS POSITIVELY SELECTED TO HETEROLOGOUS ERYTHROCYTES IN IRRADIATED PARENTAL STRAIN MICE

I. Failure to Collaborate with B Cells of the Opposite Parental Strain Not Associated with Active Suppression*

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Several studies have shown that F_1 hybrids derived from parental strains differing at the major histocompatibility complex (MHC)[1] contain two distinct subpopulations of T cells, each reactive to antigen presented in the context of one of the two parental strains. This has been demonstrated with respect to a variety of T-cell functions, e.g., measurement of proliferative responses to antigen-pulsed macrophages in vitro (1, 2), elicitation of delayed type hypersensitivity (DTH) on adoptive transfer (3), and expression of cell-mediated lympholysis (CML) directed against viruses (4), haptens (5), and minor histocompatibility determinants (6). With respect to two of these functions – T-cell proliferation and expression of DTH – the response involves T cells with the Ly 1^+ 2^- 3^- phenotype (7, and R. H. Schwartz, personal communication). This phenotype is also expressed by helper T cells involved in T-B collaboration (8).

Recent evidence suggests that macrophages play a critical role in presenting antigen in an immunogenic form to Ly 1^+ 2^- 3^- T cells. The precise contribution of macrophages in antigen presentation is not clear, although with certain antigens it has been observed that T-cell activation depends upon the responding T cells and macrophages sharing MHC determinants. In this respect, studies of Erb and Feldmann (9-11) suggest that, in vitro, mouse macrophages process antigen and complex it to small particles coded for by the *I-A* subregion of the *H-2* complex. These complexes stimulated T cells to express helper activity, but only when the T cells and the macrophages forming the complex were I-A-compatible. It is perhaps tempting to conclude from these data that all helper T cells, and perhaps Ly 1^+ 2^- 3^- cells in general, are unable to recognize antigen unless it has been processed by MHC-compatible macrophages. At present, this generalization seems premature because it fails to explain why certain antigens, e.g., particulate antigens such as heterologous erythrocytes or keyhole limpet hemocyanin cross-linked to Sepharose beads, are able to induce helper function in vitro either in the virtual absence of macrophages (12) or in the presence of MHC-incompatible macrophages (9).

The purpose of the studies presented in these two papers was to determine whether or

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* Supported by grants AI-10961 and CA-15822 from the U. S. Public Health Service.

[1] *Abbreviations used in this paper*: CML, cell-mediated lympholysis; DTH, delayed-type hypersensitivity; HRC, horse red blood cells; LN, lymph nodes; MHC, major histocompatibility complex; PE, peritoneal exudate; PFC, plaque-forming cells; SRC, sheep red blood cells; TDL, thoracic duct lymphocytes.

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not the reported dichotomy in the reactivity of F_1 hybrid T cells involved in such functions as CML and DTH also applies to helper T cells involved in T-B collaboration. This question was approached by activating purified F_1 T cells to heterologous erythrocytes in irradiated mice of one parental strain, and then harvesting the cells at a time (5 days) when the antigen-reactive precursors were presumed to have undergone clonal expansion (positive selection) after proliferating in response to the antigen in such organs as the spleen (13). The helper function of the activated T cells was then assessed in terms of their capacity to collaborate with parental strain B cells. The results show that despite the particulate nature of the antigen used, F_1 T cells exposed to sheep or horse erythrocytes in irradiated mice of one parental strain developed excellent helper activity for B cells derived from this strain, but gave only minimal help for B cells of the opposite strain. In contrast to the results of an analogous study by Skidmore and Katz (14), good collaboration was observed with F_1 B cells, and there was no evidence that suppressor cells accounted for the phenomenon.

Materials and Methods

Mice. For most experiments CBA/Cum (CBA) ($H\text{-}2^k$), C57BL/6 Cum (B6) ($H\text{-}2^b$), and (CBA × B6)F_1 hybrids were used. These mice were obtained from Cumberland View Farms, Clinton, Tenn. CBA/J mice obtained from The Jackson Laboratory, Bar Harbor, Maine were also used in some experiments. CBA/J and CBA/Cum failed to respond to each other in mixed lymphocyte culture, and were therefore assumed to be H-2- and *Mls*-identical (Mls^d). Results obtained with B cells derived from CBA/J compared with CBA/Cum mice were not discernibly different. DBA/2 ($H\text{-}2^d$) and (DBA/2 × B6)F_1 mice, also obtained from Cumberland View Farms, were used in one experiment.

Media. RPMI-1640 (Microbiological Associates, Walkersville, Md.) supplemented with 10% fetal calf serum (Microbiological Associates) was used.

Injections. All suspensions of lymphoid cells and sheep erythrocytes (SRC) and horse erythrocytes (HRC) were given intravenously unless stated otherwise.

Cells. Thoracic duct lymphocytes (TDL) were obtained as described elsewhere (15). Suspensions of spleen and lymph node (LN) cells were prepared by teasing the organs with fine forceps through an 80-mesh stainless steel sieve in cold medium.

Irradiation. Mice were exposed to ^{137}Cs γ-irradiation at a dose rate of 106 rads/min.

Cell Identification with Alloantisera. CBA anti-B6 and B6 anti-CBA *H-2* alloantisera and anti-thy 1.2 (AKR anti-C3H thymus) antiserum were prepared as described previously (16). These antisera, all of which were shown to be specific before use, were employed to establish the identity of the cells in the central lymph of irradiated B6 or CBA mice given (CBA × B6)F_1 T cells plus SRC 5 days before. In the experiment in which (DBA/2 × B6)F_1 T cells were activated in irradiated DBA/2 and B6 mice, DBA/2 anti-B6 and B6 anti-DBA/2 alloantisera were used; these sera were prepared as referred to above. 50 μl of the test lymphocytes (5×10^6/ml) were incubated at 4°C for 30 min with 25 μl of antisera in small plastic tubes and washed once by centrifugation. The cell pellet was resuspended in 50 μl of complement (1:5 dilution of guinea pig serum) for 30 min at 37°C. Cytotoxic indices were then calculated by dye exclusion with respect to controls incubated with normal mouse serum plus complement or with antiserum without complement. Percent lysis with the control samples was invariably <5%. The anti-thy 1.2 serum appeared to be specific for T cells, since it lysed 97–100% of LN cells filtered from blood to lymph through irradiated mice (16), 20–30% of spleen cells, and <5% of bone marrow cells.

Preparation of T Cells for Positive Selection. The T cells used for positive selection were obtained from pooled mesenteric, inguinal, axillary, and cervical LN of unprimed (CBA × B6)F_1 or (DBA × B6)F_1 mice. LN cells were depleted of most thy 1.2-negative cells by passage over nylon-wool columns as described by Julius et al (17). The majority of the effluent cells recovered from the columns were T cells, since 86–95% (mean - 91%, 15 experiments) were susceptible to lysis by anti-thy 1.2 antiserum in the presence of complement. The recovery of T cells after nylon-wool passage was usually on the order of 60–80% (established with respect to the number of thy 1.2-positive cells in the LN cell preparation before passage [50–70%]). The viability of the cells recovered from the column was usually >85%.

Positive Selection to Heterologous Erythrocytes in Irradiated Mice. In most experiments, 5 × 10^7 viable, unprimed, nylon-wool-passed (CBA × B6)F_1 T cells in a 0.5-ml volume were mixed with 0.5 ml of 25% SRC (or HRC) and injected intravenously into CBA, B6, or (CBA × B6)F_1 mice (2–5 mice per group) given 800 rads 1 day previously. (DBA/2 × B6)F_1 T cells were used in one experiment, and these cells were transferred with SRC into irradiated B6 or DBA/2 mice. Thoracic duct fistulas were inserted in the mice 5 days later. TDL were collected overnight and used as helper T cells after establishing their identity with appropriate alloantisera.

Preparation of B Cells. Spleen cells from mice primed with SRC or with both SRC and HRC were resuspended in undiluted anti-thy 1.2 antiserum (0.1 ml of antisera/2 × 10^7 spleen cells) and kept at 4°C for 30 min. After washing twice by centrifugation, the cells were incubated at 37°C for 30 min with guinea pig complement (1:6 dilution, 2 × 10^7 cells/ml). The cells were then washed once and resuspended to a volume suitable for injection. This treatment lysed 20–45% of the spleen cells.

Measurement of T-B Collaboration. In most experiments, helper T cells (usually 0.8 × 10^6 TDL from irradiated mice given F_1 T cells plus SRC 5 days before) were transferred with 0.1 ml of 5% SRC into irradiated (750 rads 1 day before) F_1 hybrid mice with B cells (anti-thy 1.2-treated spleen cells) prepared from SRC-primed F_1 hybrid or parental strain mice. With one exception, B cells were transferred in a dose of 5 × 10^6 viable cells. The exception occurred when B6 B cells were transferred to irradiated (CBA × B6)F_1 recipients; here, presumably because of the strong *Hh* barrier existing in this situation (18), a higher dose of cells (8–10 × 10^6) had to be transferred to get responses equivalent to those given by the other B-cell populations. In experiments in which responses to HRC were measured, both SRC and HRC (0.1 ml of 5% of each) were added to the injected mixture of T and B cells; in this situation, the B cells were prepared from mice primed to both SRC and HRC.

Plaque-Forming Cell (PFC) Assays. Direct (19s, IgM) PFC were detected by the method of Cunningham and Szenberg (19). Numbers of indirect (7s, IgG) PFC were measured by adding a polyvalent rabbit anti-mouse immunoglobulin reagent to the reactive mixture in the presence of specific goat anti-mouse μ-chain serum. As described elsewhere (16), the anti-μ serum (kindly provided by Dr. M. Feldmann, University College, London, England) suppressed IgM but not IgG PFC.

Serum Hemagglutinin Assays. These were performed by the method of Dietrich (20).

Priming with Heterologous Erythrocytes. SRC and HRC (obtained from Gibco Diagnostics, The Mogul Corp., Chagrin Falls, Ohio) were stored in Alsever's solution and washing three times before use. Mice were primed intraperitoneally with 0.1 ml of 25% SRC (or also with a similar dose of HRC) and used as B-cell donors 2–4 mo later.

Peritoneal Exudate (PE) Cells. PE cells were obtained from mice given a 1-ml intraperitoneal injection of 4% thyoglycollate solution (Difco Laboratories, Detroit, Mich.) 4 days earlier. The cells were treated with anti-thy 1.2 serum and complement according to the procedure used for preparing B cells from spleen.

Statistical Analysis. Geometric means and values used to derive the upper and lower limits of the SE of the mean (these values represent the anti-log of SE of $\log_{10}$ geometric mean) were calculated from the $\log_{10}$ of the PFC counts. Arithmetic means and standard errors of the mean were calculated from the reciprocal of the $\log_2$ values for the hemagglutinin titers. *P* values were determined by Student's *t* test. In the comparison of the means of any two groups of observations, a significance level of 0.05 was chosen.

Results

Experimental Design. The general plan of the experiments was to transfer LN T cells from unprimed (CBA × B6)F_1 mice into irradiated CBA, B6, and (CBA × B6)F_1 mice together with SRC, and then to study the helper function of the SRC-activated T cells recovered from the thoracic duct lymph of the recipients 5 days later. Before transfer, the LN cells were depleted of B cells (and presumably most macrophages) by nylon-wool filtration. The effluent T cells (>90% thy-1.2-positive) were transferred intravenously in a dose of 5 × 10^7 cells with SRC (0.5 ml of 25% solution), also given intravenously. To limit the

TABLE I
Identity of TDL Recovered from Thoracic Duct Lymph of Irradiated CBA, B6, and (CBA × B6)F_1 Mice Injected with Unprimed Nylon-Wool-Purified (CBA × B6)F_1 LN Cells Plus SRC 5 Days Previously

Recipients of (CBA × B6)F_1 T cells plus SRC*	Average no. of TDL (× 10^{-6}) collected from each recipient over 18 h‡	Cytotoxic index with alloantisera + complement§			Host origin
		B6 anti-CBA	CBA anti-B6	Anti-thy 1.2	
					%
Irrad. CBA	14.5	99	95	97	5
Irrad. B6	12.1	95	100	98	4
Irrad. (CBA × B6)F_1	14.3	99	99	97	–

* Nylon-wool-purified LN T cells (93% thy 1.2-positive) were transferred intravenously in a dose of 5 × 10^7 viable cells with 0.5 ml of 25% SRC; the cell recipients (three mice per group) were exposed to 800 rads 1 day before.

‡ Thoracic duct fistulas were inserted at 5 days after cell transfer. TDL were collected overnight, the collections commencing at about 4 h after establishing the fistulas. Lymph samples were pooled from the three mice in each group before counting.

§ See Materials and Methods. Background lysis with cells treated with normal mouse serum plus complement was <3%.

possibility of a host-versus-graft reaction, the transfer hosts received irradiation at a high dose (800 rads) 1 day before cell transfer. Thoracic duct fistulas were inserted in the cell recipients at 5 days post-transfer, i.e. during the stage of positive selection when it was presumed that the progeny of the cells responding to the injected antigen in the lymphoid tissues entered the circulation in large numbers (21, 22). The features of the lymph-borne cells collected over a 16-h period are shown in Table I; the data are from one experiment which is representative of many others. Testing with anti-thy 1.2 serum and appropriate alloantisera in the presence of complement showed that the lymph-borne cells consisted almost entirely (≥97%) of T cells, nearly all of which were of donor origin (>94%). Cell viability was 99–100%. The yield of cells (compared with the numbers initially injected) was high (≃30%).

To study their helper function, the cells were transferred intravenously in small numbers (0.8 × 10^6) into irradiated (750 rads) (CBA × B6)F_1 mice, together with SRC (0.1 ml of 5% solution), and B cells (5–8 × 10^6 anti-thy 1.2-serum-treated spleen cells from SRC-primed mice). Numbers of direct (IgM) and indirect (IgG) PFC to SRC were measured in the spleen 7 days later.

As shown in Table II, (CBA × B6)F_1 T cells activated to SRC in irradiated (CBA × B6)F_1 mice (F_1 $T_{+(SRC-F_1)}$), provided effective help for B cells derived from either of the two parental strains, as well as for F_1 B cells. By contrast, F_1 T cells activated to SRC in irradiated B6 mice (F_1 $T_{+(SRC-B6)}$) or in CBA mice (F_1 $T_{+(SRC-CBA)}$) cooperated well with B cells derived from only one of the two parental strains, namely the strain in which the T cells were initially activated. The failure to stimulate more than minimal responses by B cells of the opposite parental strain did not seem to be the result of active suppression, since a supplemental injection of F_1 $T_{+(SRC-F_1)}$ cells led to high responses. Moreover,

TABLE II

Helper Activity of T Cells Recovered from Thoracic Duct Lymph of Irradiated CBA, B6, and (CBA × B6)F_1 Mice Injected 5 Days Previously with Unprimed (CBA × B6)F_1 T Cells Plus SRC[*]

T-cell group	T cells (0.8 × 10^6)[‡]	B cells[§]	Anti-SRC PFC/spleen at 7 days in irradiated F_1 mice	
			IgM	IgG
		CBA	1,150 (1.08)[‖]	8,610 (1.01)
A	F_1 $T_{+(SRC\text{-}B6)}$	B6	40, 590 (1.13)	118,870 (1.15)
		(CBA × B6)F_1	7,370 (1.28)	84,660 (1.16)
		CBA	30,490 (1.02)	275,400 (1.17)
B	F_1 $T_{+(SRC\text{-}CBA)}$	B6	3,940 (1.05)	9,360 (1.15)
		(CBA × B6)F_1	13,060 (1.13)	74,500 (1.11)
		CBA	21,190 (1.25)	125,690 (1.08)
C	F_1 $T_{+(SRC\text{-}F_1)}$	B6	13,890 (1.18)	56,180 (1.14)
		(CBA × B6)F_1	14,680 (1.16)	89,360 (1.06)
Group A + group C		CBA	19,170 (1.30)	131,390 (1.25)
Group B + group C		B6	20,380 (1.23)	64,960 (1.27)
–		CBA	200 (1.40)	510 (1.07)
–		B6	990 (1.39)	1,740 (1.26)
–		(CBA × B6)F_1	<100	610 (1.22)
Group A		–	<100	<100
Group B		–	<100	<100
Group C		–	<100	<100

[*] Cell recipients were exposed to 800 rads 1 day before being injected intravenously with 5 × 10^7 unprimed nylon-wool-purified (CBA × B6)F_1 T cells plus 0.5 ml of 25% SRC.

[‡] Recipients of F_1 T cells and SRC were cannulated at 5 days post-transfer and the lymph-borne cells were collected overnight. The helper activity of the cells was measured by transferring 0.8 × 10^6 TDL (nearly all of which were T cells of F_1 origin; Table I) intravenously into irradiated (750 rads) (CBA × B6)F_1 mice with B cells and 0.1 ml of 5% SRC. When transferring mixtures of T cells to check for suppression, a dose of 0.8 × 10^6 of each T-cell population was used.

[§] Anti-thy 1.2-treated spleen cells from mice primed with SRC 2-4 mo previously; 5 × 10^6 (CBA and (CBA × B6)F_1) or 8 × 10^6 (B6) viable cells transferred.

[‖] Geometric mean of data from four mice per group (two mice only for T cells transferred without B cells); number in parenthesis refers to value by which mean is multiplied by or divided to give upper and lower limits, respectively, of SE. All values shown are significantly above ($P < 0.05$) the background values of B cells transferred without T cells.

effective cooperation was observed with F_1 B cells. Five other experiments gave similar results.

Unless stated otherwise, the design of the experiments to be considered below was identical to the protocol used in Table II. In all experiments, the helper cells were recovered from thoracic duct lymph at day 5–6 post-transfer, and they were pooled from at least 2 mice per group. In each experiment the identity of the lymph-borne cells was checked with anti-thy 1.2 serum and appropriate *H-2* alloantisera. By these parameters, the lymph-borne cells were invariably >90% (and usually >95%) T cells of donor F_1 origin. For simplicity, the background values obtained when B cells were transferred without T cells have been subtracted from the data shown in Tables III–VIII. These values were generally

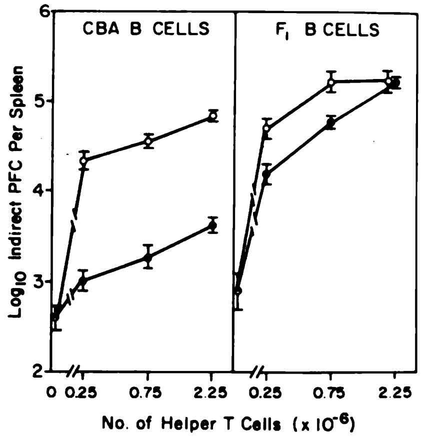

FIG. 1. Dose response of helper activity of (CBA × B6)F_1 T cells activated to SRC in irradiated B6 (●) or irradiated (CBA × B6)F_1 (○) mice and harvested from thoracic duct lymph of the recipients 5 days later. The fig. shows numbers of anti-SRC IgG PFC/spleen in irradiated (CBA × B6)F_1 mice injected intravenously 7 days previously with varying doses of one of the two T-cell populations plus 0.1 ml of 5% SRC and either CBA B cells or (CBA × B6)F_1 B cells. ◓, B cells transferred without T cells; PFC numbers with T cells transferred without B cells were < 100 PFC/spleen. The B cells (5 × 10^6 viable anti-thy 1.2-serum-treated spleen cells) were from mice primed with SRC 2 mo previously. Each point represents geometric mean of data from four mice; vertical bars show upper and lower limits of SE.

quite low (200–2,000 PFC/spleen) and are shown in the footnotes to the tables. T cells transferred without B cells gave negligible responses (<200 PFC/spleen). It should be stressed that the irradiated recipients used for measuring T-B collaboration were invariably F_1 hybrids, i.e. mice syngeneic or semi-syngeneic with the T and B cells.

Effect of Varying the Dose of Helper T Cells. Although the helper function of F_1 T cells activated to SRC in mice of one parental strain was very low for B cells of the opposite parental strain, it was significant. This is illustrated in Fig. 1 where it can be seen that F_1 $T_{+(SRC-B6)}$ cells gave a linear response when transferred in varying doses with a constant number of CBA B cells. With the highest dose of T cells (2.25 × 10^6), the response was 20-fold above background. It is apparent that the slope of the dose-response curve obtained in this situation paralleled the slope of the (far greater) response observed when CBA B cells were transferred with varying doses of F_1 $T_{+(SRC-F_1)}$ cells.

In the case of responses with F_1 B cells, it is of interest that the helper activity of F_1 $T_{+(SRC-B6)}$ cells was appreciably less than that of F_1 $T_{+(SRC-F1)}$ cells; this was apparent where low doses of T cells were transferred, though not with high doses. The significance of this curious finding with low T-cell doses (which is also evident in Table IV, V, and Fig. 2) will be considered in the following paper.

Effect of Transferring F_1 T Cells to Irradiated Parental Strain Mice with or without Antigen. When (CBA × B6)F_1 T cells were transferred to irradiated B6 mice without SRC, the lymph-borne cells collected at day 5 (F_1 T_{+B6}) gave significant though small anti-SRC and anti-HRC responses with CBA, B6, and

TABLE III

*Helper Activity of T Cells Recovered from Thoracic Duct Lymph of Irradiated B6 Mice Injected 5 Days Previously With Unprimed (CBA × B6)F_1 T Cells Given with or without SRC**

T-cell group	T cells	Dose of T cells ($\times 10^{-6}$)	B cells‡	PFC/spleen at 7 days in irradiated F_1 mice		
				Anti-SRC		Anti-HRC
				IgM	IgG	IgM
A	F_1 T_{-B6}	0.4	CBA	1,180 (1.29)§	1,390 (1.29)‖	20 (1.65)‖
		2.0	CBA	4,210 (1.12)	10,760 (1.20)	830 (1.30)
		0.4	B6	1,300 (1.55)‖	1,930 (1.34)‖	1,250 (1.58)‖
		2.0	B6	3,560 (1.25)	5,680 (1.19)	3,690 (1.20)
		0.4	(CBA × B6)F_1	150 (1.09)‖	3,350 (1.15)	140 (1.46)‖
		2.0	(CBA × B6)F_1	4,680 (1.15)	21,870 (1.18)	4,510 (1.23)
B	F_1 $T_{+(SRC-B6)}$	0.4	CBA	1,620 (1.21)	1,550 (1.06)‖	0
		2.0	CBA	4,750 (1.25)	12,420 (1.25)	1,180 (1.20)
		0.4	B6	26,340 (1.20)	66,050 (1.12)	1,750 (1.23)
		2.0	B6	55,060 (1.23)	100,440 (1.13)	6,950 (1.19)
		0.4	(CBA × B6)F_1	8,140 (1.25)	35,900 (1.24)	640 (1.43)‖
		2.0	(CBA × B6)F_1	58,130 (1.36)	188,680 (1.28)	6,540 (1.53)

* As for footnote to Table II; group A. T cells were TDL from irradiated B6 mice given 5×10^7 unprimed (CBA × B6)F_1 T cells, but not SRC, 5 days previously; group B. T cells were from irradiated B6 mice given F_1 T cells with SRC (0.5 ml of 25%) 5 days before.

‡ As for footnote to Table II except that the B cells were taken from mice primed with both SRC and HRC.

§ As for footnote to Table II except that background values given by B cells transferred without T cells have been subtracted. These values (PFC/spleen) were: CBA 950(1.18) (IgM SRC), 2,050(1.36) (IgG SRC), 440(1.41) (IgM HRC); B6 850(1.50) (IgM SRC), 1,890(1.70) (IgG SRC), 430(1.45) (IgM HRC); (CBA × B6)F_1 1,000(1.17) (IgM SRC), 2,840(1.19) (IgG SRC), 870(1.36) (IgM HRC). Values for T cells transferred without B cells were all <100 PFC/spleen. In the table values in parentheses used to derive limits of SE (see footnote to Table II) were calculated before subtraction of the background PFC numbers.

‖ Not significantly above background values of B cells transferred alone ($P > 0.05$).

F_1 B cells (Table III). These responses were dose-dependent, i.e. 2×10^6 T cells produced moderate numbers of PFC, whereas 0.4×10^6 T cells gave responses which were generally not significantly higher than the background values observed when B cells alone were transferred.

Markedly different results occurred when SRC were added to the F_1 T cells transferred to the irradiated B6 mice. Three points can be made concerning the helper activity of these cells. First, even small doses of these cells (F_1 $T_{+(SRC-B6)}$) gave high anti-SRC responses with B6 and F_1 B cells, though not with CBA B cells. Second, the low response observed with CBA B cells was similar in magnitude to that given by T cells from recipients not given SRC (F_1 T_{+B6} cells). Third, the response to a different antigen, HRC, was low with all three populations of B cells.

Specificity of Positive Selection. To obtain further information on the specificity of the F_1 T cells positively selected to SRC in the above experiments, F_1 T cells were activated in (*a*) irradiated B6 mice given SRC (F_1 $T_{+(SRC-B6)}$), (*b*) irradiated B6 mice given HRC (F_1 $T_{+(HRC-B6)}$), and (*c*) irradiated F_1 mice given

TABLE IV

Helper Activity of T Cells Recovered from Thoracic Duct Lymph of Irradiated B6 Mice Injected 5 Days Previously with (CBA × B6)F_1 T Cells Plus Either SRC or HRC[*]

T-cell group	T cells (0.8 × 10^6)[‡]	B cells[§]	IgG PFC/spleen at 7 days in irradiated F_1 mice	
			Anti-SRC	Anti-HRC
A	F_1 $T_{+(\mathrm{SRC-B6})}$	CBA	960 (1.27)[‖]	1,160 (1.28)
		B6	40,770 (1.28)	5,830 (1.08)
		(CBA × B6)F_1	16,360 (1.15)	3,180 (1.16)
B	F_1 $T_{+(\mathrm{HRC-B6})}$	CBA	460 (1.23)[¶]	940 (1.53)[¶]
		B6	1,940 (1.37)	61,420 (1.13)
		(CBA × B6)F_1	2,500 (1.13)	32,110 (1.09)
C	F_1 $T_{+(\mathrm{SRC+HRC-F_1})}$	CBA	35,310 (1.14)	26,550 (1.17)
		B6	12,390 (1.24)	29,400 (1.26)
		(CBA × B6)F_1	42,360 (1.13)	44,990 (1.18)

[*] As for footnote to Table II; T cells were taken from lymph of irradiated B6 mice given, 5 days previously, 5 × 10^7 unprimed (CBA × B6)F_1 T cells with 0.5 ml of 25% SRC (group A) or HRC (group B); group C. T cells were from irradiated (CBA × B6)F_1 mice given F_1 T cells together with both SRC and HRC.

[‡] As for footnote to Table II except that the T and B cells were transferred with both SRC and HRC (0.1 ml of 5% of each).

[§] As for footnote to Table II except that B cells were prepared from mice primed with both SRC and HRC.

[‖] As for footnote to Table II except that background values given by B cells transferred without T cells have been subtracted. These values (PFC/spleen) were: CBA 350(1.45) (SRC), 470(1.33) (HRC); B6 1,000(1.26) (SRC), 800(1.19) (HRC); (CBA × B6)F_1 950(1.36) (SRC), 290(1.07) (HRC). In the table the values in parentheses used to derive limits of SE (see footnote to Table II) were calculated before subtraction of the background PFC numbers. Numbers of PFC for T cells transferred without B cells were all <100/spleen.

[¶] Not significantly above background values of B cells transferred alone ($P > 0.05$).

both SRC and HRC (F_1 $T_{+(\mathrm{SRC+HRC-B6})}$). The helper function of these three groups of cells is shown in Table IV. In the case of F_1 $T_{+(\mathrm{SRC-B6})}$ cells, anti-SRC responses were high with B6 and F_1 B cells but not with CBA B cells; anti-HRC responses were low with all three B-cell populations. Reciprocal results were found with F_1 $T_{+(\mathrm{HRC-B6})}$ cells, i.e. anti-SRC responses were all low, whereas anti-HRC responses were high with B6 and F_1 B cells, but not with CBA B cells. F_1 $T_{+(\mathrm{SRC+HRC-B6})}$ cells stimulated all three B-cell populations to produce high responses to both SRC and HRC.

Time of PFC Assay. In the preceding experiments, numbers of PFC were measured arbitrarily on day 7 post-transfer. Fig. 2 shows that essentially similar results occurred when PFC were assayed on days 5, 7, and 9. Thus, at no stage of assay did F_1 $T_{+(\mathrm{SRC-B6})}$ cells give > 1,800 anti-SRC PFC/spleen with CBA B cells. By contrast, F_1 $T_{+(\mathrm{SRC-F1})}$ cells elicited high responses with CBA B cells, PFC numbers reaching a peak on day 7 (46,730 IgG PFC), and then declining.

Serum Hemagglutinin Titers. It might be argued that despite the failure of F_1 $T_{+(\mathrm{SRC-B6})}$ cells to stimulate CBA B cells to differentiate into PFC in the spleen, effective collaboration could have occurred in other regions. If so, this

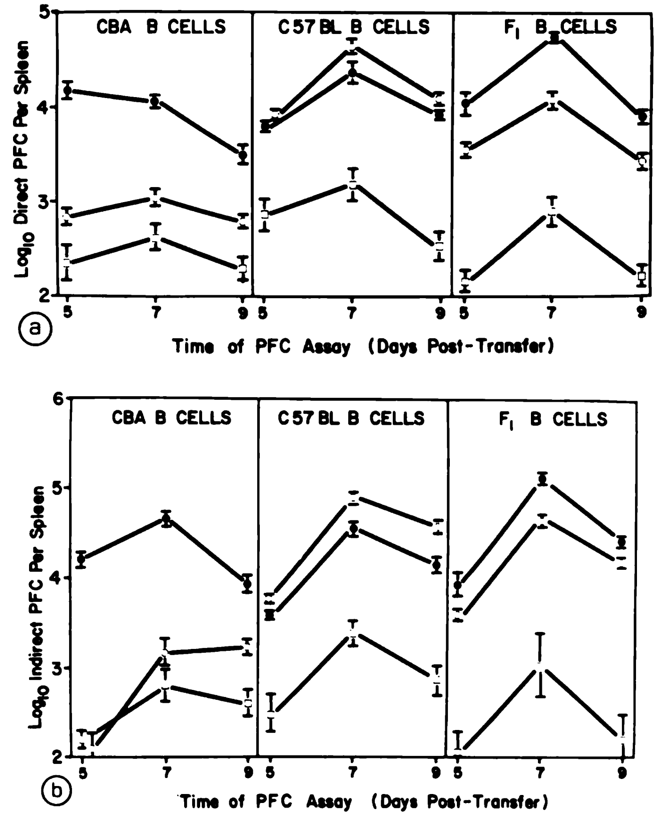

FIG. 2. Time response of helper activity of (CBA × B6)F_1 T cells activated to SRC in irradiated B6 (○) or (CBA × B6)F_1 (●) mice and harvested from thoracic duct lymph of the recipients 5 days later. The fig. shows numbers of anti-SRC IgM (direct) (a) and IgG (indirect) (b) PFC/spleen in irradiated (CBA × B6)F_1 mice at various times after intravenous injection of one of the two T-cell populations (0.8×10^6 cells) plus 0.1 ml of 5% SRC and B cells from CBA, B6 (C57BL), or (CBA × B6)F_1 mice. □, B cells transferred without T cells. The B cells (anti-thy 1.2-serum-treated spleen cells) were from SRC-primed mice and were transferred in a dose of 5×10^6 viable cells (8×10^6 for B6 B cells). Number of PFC with T cells transferred without B cells was < 100 PFC/spleen at all time points. Each point represents geometric mean of data from four mice; vertical bars show upper and lower limits of SE.

would presumably be reflected in the levels of serum hemagglutinins. To investigate this possibility, the mice used for PFC assay at day 9 in the above experiment (Fig. 2) were exsanguinated at the time of sacrifice, and these sera were measured for anti-SRC hemagglutinin activity. As shown in Table V, the serum hemagglutinin titers closely reflected the results obtained by measuring numbers of PFC in the spleen (compare with Fig. 2).

Effect of Adding PE Cells during Positive Selection. Macrophages present in the irradiated parental strain mice used for positive selection might be responsible for the restricted helper function observed after selection. If so, the restriction would be expected not to occur if the mice used for selection were supplemented with macrophages of the opposite parental strain. To investigate

TABLE V

*Helper Activity of T Cells Recovered from Thoracic Duct Lymph of Irradiated B6 and (CBA × B6)F_1 Mice Injected 5 Days Previously with (CBA × B6)F_1 T Cells and SRC: Serum Hemagglutinin Titers after Transfer of T and B Cells to Irradiated (CBA × B6)F_1 Mice**

T cells (0.8 × 10^6)	B cells	Anti-SRC hemagglutinin titers (1/$\log_2$) at 9 days after transfer to irradiated F_1 mice
F_1 $T_{\rightarrow(SRC\rightarrow B6)}$	CBA	1.6 (±0.8)‡
	B6	5.7 (±0.5)
	(CBA × B6)F_1	6.7 (±0.5)
F_1 $T_{\rightarrow(SRC\rightarrow F_1)}$	CBA	6.1 (±0.9)
	B6	4.8 (±0.8)
	(CBA × B6)F_1	9.4 (±1.1)

* For details see legend to Fig. 2. Sera tested were from mice assayed for splenic PFC at day 9 after transfer of T and B cells to irradiated (CBA × B6)F_1 mice; the numbers of PFC for these mice are shown in Fig. 2.

‡ Arithmetic mean (±SE) after subtraction of background values given by B cells transferred without T cells. These values were: CBA 1.9 (±0.5); B6 3.6 (±0.9); (CBA × B6)F_1 2.9 (±0.7). SE were calculated before background subtraction. Titers from mice given T cells but not B cells were <1.4 mice per group. The value asterisked is not significantly above background value of B cells transferred alone; all other values are significantly above background values ($P < 0.05$).

this possibility, (CBA × B6)F_1 T cells were positively selected to SRC in irradiated B6 mice injected with 3 × 10^7 anti-thy 1.2-serum-treated PE cells derived from either CBA or B6 mice (groups B, C; Table VI); of these cells, >80% had features of macrophages, i.e. they were large cells and contained numerous granules. The F_1 T cells and SRC were mixed with the PE cells and injected intraperitoneally; attempts to transfer this mixture intravenously caused respiratory distress and rapid death, presumably because of clumping of the PE cells. For controls, F_1 T cells and SRC were transferred intravenously into irradiated B6 and CBA mice (groups A, D; Table VI).

The helper activity of TDL recovered from these four groups of mice at day 5 post-transfer is shown in Table VI. It is apparent that addition of CBA PE cells during positive selection in irradiated B6 mice did not lead to restriction in helper function, i.e. the TDL recovered from these mice collaborated well with either CBA or B6 B cells. By contrast, TDL recovered after positive selection in the presence of B6 PE cells led to high responses with B6 B cells, but not with CBA B cells. With respect to the helper cells recovered from Group B, it should be mentioned that >98% of the cells were susceptible to lysis with either anti-thy 1.2 serum or CBA anti-B6 alloantiserum. Thus, there was no evidence that the CBA PE cells given to these mice reached the central lymph.

Positive Selection with (DBA/2 × B6)F_1 T Cells. To determine whether or not the above findings with (CBA × B6)F_1 T cells also applied to other strain combinations, (DBA/2 × B6)F_1 T cells were activated to SRC in irradiated DBA/

TABLE VI

*Helper Activity of T Cells Recovered from Thoracic Duct Lymph of Irradiated B6 Mice Injected 5 Days Previously with Unprimed (CBA × B6)F_1 T Cells Plus CBA PE Cells and SRC**

T cell group	T cells (0.8×10^6)‡	PE cells added during positive selection	B cells§	Anti-SRC PFC/spleen at 7 days in irradiated F_1	
				IgM	IgG
A	F_1 $T_{+(SRC-B6)}$	–	CBA	1,770 (1.12)‖	1,580 (1.52)¶
			B6	17,280 (1.14)	37,050 (1.13)
B	F_1 $T_{+(SRC-B6)}$	3×10^7 CBA PE	CBA	26,950 (1.16)	67,740 (1.13)
			B6	21,160 (1.19)	47,880 (1.16)
C	F_1 $T_{+(SRC-B6)}$	3×10^7 B6 PE	CBA	2,150 (1.34)	8,140 (1.37)
			B6	57,330 (1.24)	114,910 (1.02)
D	F_1 $T_{+(SRC-CBA)}$	–	CBA	19,740 (1.12)	59,400 (1.06)
			B6	1,300 (1.30)	2,160 (1.14)

* As for footnote to Table II except that in the case of groups B and C, the F_1 T cells (5×10^7 nylon-wool-passed), PE cells and SRC were all injected intraperitoneally in one injection; in groups A and D, the F_1 T cells and SRC were transferred intravenously. The PE cells were from mice given thyoglycolate 4 days before and were treated with anti-thy 1.2 antiserum and complement before injection.

§ As for footnote to Table II.

‖ As for footnote to Table II except that background values given by B cells transferred without T cells have been subtracted. These values (PFC/spleen) were: CBA 190(1.57) (IgM), 700(1.76) (IgG); B6 130(1.44) (IgM), 370(1.46) (IgG). Values for T cells transferred without B cells were all <400 PFC/spleen. In the table values in parentheses used to derive limits of SE (see footnote to Table II) were calculated before subtraction of background PFC numbers.

¶ Not significantly above background value of B cells transferred without T cells ($P > 0.05$).

2 or B6 mice. Their helper activity was then assayed in irradiated (DBA/2 × B6)F_1 mice. As shown in Table VII, F_1 $T_{+(SRC-DBA/2)}$ cells collaborated well with DBA/2 and F_1 B cells, but not with B6 B cells. By contrast, F_1 $T_{+(SRC-B6)}$ cells stimulated B6 and F_1 B cells, but not DBA/2 B cells. Injection of both populations of T cells with either of the two parental strain B-cell populations led to high responses, i.e. suppression was not observed.

Positive Selection Using Activated T Cells Recovered from the Spleen. All of the preceding experiments involved the use of activated helper cells recovered from thoracic duct lymph of the intermediate irradiated hosts. Two experiments were also performed with helper cells recovered from the spleen. Cells from this region were less satisfactory to work with because of their low viability (20–40%) and greater contamination with radioresistant host cells (15–30%). Nevertheless, the qualitative helper activity of these cells was similar to that of the cells obtained from the central lymph. Thus, it can be seen in Table VIII that (CBA × B6)F_1 T cells positively selected to SRC in irradiated B6 mice and recovered from the spleen of the recipients 5 days later collaborated well with (CBA × B6)F_1 B cells, but poorly with CBA B cells. By contrast, F_1 T cells activated in irradiated F_1 mice stimulated both CBA and F_1 B cells. Mixtures of these two T-cell populations gave high responses with CBA B cells.

TABLE VII

*Helper Activity of T Cells Recovered from Thoracic Duct Lymph of Irradiated B6 and DBA/2 Mice Injected 5 Days Previously with Unprimed (DBA/2 × B6)F_1 T Cells Plus SRC**

T-cell group	T cells (0.8×10^6)‡	B cells§	Anti-SRC PFC/spleen at 7 days in irradiated (DBA/2 × B6)F_1 mice	
			IgM	IgG
A	F_1 $T_{\rightarrow(SRC-B6)}$	B6	17,790 (1.13)‖	34,400 (1.26)
		DBA/2	310 (1.18)	390 (1.60)
		(DBA/2 × B6)F_1	7,840 (1.23)	35,860 (1.09)
B	F_1 $T_{\rightarrow(SRC-DBA/2)}$	B6	390 (1.38)	250 (1.28)
		DBA/2	4,340 (1.05)	17,990 (1.10)
		(DBA/2 × B6)F_1	9,360 (1.14)	29,010 (1.02)
	Group A + group B	B6	17,980 (1.48)	32,690 (1.35)
	Group A + group B	DBA/2	3,730 (1.27)	18,180 (1.14)

* Cell recipients were exposed to 800 rads 1 day before being injected intravenously with 5×10^7 unprimed nylon-wool-purified (DBA/2 × B6)F_1 T cells plus 0.5 ml of 25% SRC.

‡ As for footnote to Table II except that irradiated (DBA/2 × B6)F_1 mice were used as the recipients of the T and B cells. When transferring a mixture of group A and group B cells to check for suppression, a dose of 0.8×10^6 of each T-cell population was used.

§ Anti-thy 1.2-treated spleen cells from mice primed with SRC 3 mo previously; 5×10^6 (DBA/2 and (DBA/2 × B6)F_1) or 8×10^6 (B6) viable cells transferred.

‖ As for footnote to Table II except that background values given by B cells transferred without T cells have been subtracted. These values (PFC/spleen) were: B6 100(1.37) (IgM), <100 (IgG); DBA/2 < 100 (IgM), 140(1.24) (IgG); F_1 160(1.30) (IgM), 340(2.27) (IgG). PFC numbers for T cells transferred without B cells were <100/spleen. In the table values in parentheses used to derive limits of SE were calculated before subtraction of background PFC numbers. All values shown in the Table are significantly above the values of B cells transferred without T cells ($P < 0.05$).

Discussion

A priori, the restriction in helper function observed in the present studies might have been due to the induction of suppressor cells during T-cell activation. In this respect it is of particular relevance that Skidmore and Katz (14), using a hapten-carrier system, recently reported that F_1 T cells activated to the carrier in irradiated mice of one parental strain stimulated anti-hapten responses by B cells of this strain, but not those of the opposite strain. In marked contrast to the present studies, however, cooperation was not observed with F_1 B cells. The authors concluded that this failure to trigger F_1 B cells reflected the presence of suppressor cells generated during T-cell activation. It was not determined whether the suppressors were of host or of donor origin and whether or not they were antigen-specific. On the basis of these findings the authors argued that F_1 T cells contain two subpopulations of helper cells, each able to collaborate with only one of the two populations of parental strain B cells. However, it is difficult to justify this reasoning since the presence of suppressor cells in their system made it impossible to determine whether the failure to observe collaboration with B cells of the opposite parental strain reflected an innate restriction in the specificity of the helper cells rather than simple destruction of either the T cells, B cells, or both by the suppressor cells.

TABLE VIII

*Helper Activity of T Cells Recovered from Spleen of Irradiated B6 and (CBA × B6)F_1 Mice Injected 5 Days Previously with Unprimed (CBA × B6)F_1 T Cells Plus SRC**

T-cell group	T cells‡ (10^6)	B cells§	Anti-SRC PFC/spleen at 7 days in irradiated F_1 mice	
			IgM	IgG
A	F_1 $T_{\rightarrow(\mathrm{SRC}-\mathrm{B6})}$	CBA	980 (1.22)‖	6,930 (1.17)
		(CBA × B6)F_1	11,890 (1.24)	60,760 (1.21)
B	F_1 $T_{\rightarrow(\mathrm{SRC}-F_1)}$	CBA	10,010 (1.13)	60,900 (1.18)
		(CBA × B6)F_1	38,580 (1.25)	108,380 (1.07)
	Group A + group B	CBA	19,280 (1.47)	85,140 (1.22)

* As for footnote to Table II.

‡ T cells were harvested from the spleens of four mice per group at 5 days after transfer of F_1 T cells plus SRC. Most of the cells recovered from the spleens were (*a*) thy 1.2-positive, i.e. 93% for group A and 90% for group B, and (*b*) of donor F_1 origin, i.e. 85% of group A cells were killed by B6 anti-CBA alloantiserum. When transferring a mixture of group A and group B cells to check for suppression, a dose of 10^6 viable cells of each T-cell population was used.

§ As for footnote to Table II.

‖ As for footnote to Table II except that background values given by B cells transferred without T cells have been subtracted. These values (PFC/spleen) were: CBA 160(1.07) (IgM), 170(1.22) (IgG); (CBA × B6)F_1 630(1.27) (IgM), 2,490(1.27) (IgG). In the table values in parentheses used to derive limits of SE (see footnote to Table II) were calculated before subtraction of background PFC numbers. Numbers of PFC for T cells transferred without B cells were all <200/spleen. All values shown in the table are significantly above the background values of B cells transferred without T cells ($P < 0.05$).

In the present studies, three lines of evidence suggest that the restriction in helper function was not due to the presence of suppressor cells. First, the failure of F_1 T cells activated to antigen in one parental strain to collaborate with B cells of the opposite strain was not infectious, i.e., high responses were invariably observed when these populations of T and B cells were transferred in the presence of F_1 T cells activated either in mice of the other parental strain or in F_1 mice (Tables II, VII, VIII). Second, F_1 T cells activated in parental strain mice cooperated well with F_1 B cells. Third, the poor responses found with B cells of the opposite parental strain, although very low in magnitude, were not insignificant. With small numbers of helper cells these responses were close to background levels but rose in a linear fashion to appreciable levels as the dose of T cells was increased (see Fig. 1). Moreover, the magnitude of these responses was comparable to the responses observed with similar doses of unprimed F_1 T cells (unpublished data) or with unprimed F_1 T cells passaged through irradiated parental strain mice without antigen (Table III).

In view of the evidence mentioned earlier that F_1 T cells contain two discrete populations of antigen-reactive cells, it would seem reasonable that such a dichotomy accounted for the present findings. If so, can the data be taken to indicate that F_1 T cells do consist of two subpopulations of helper cells, each able to collaborate with B cells derived from only one of the two parental strains? Before approaching this question it is first necessary to consider the nature of positive selection to antigen in the irradiated parental strain environment. Since the function of positively selected T cells was measured in terms of B-cell stimulation, one might argue that antigen presentation in the irradiated intermediate hosts was controlled by host B cells. This seems unlikely since B lymphocytes are exquisitely radiosensitive and die within a few hours of irradiation (23). Moreover, there is no evidence that B lymphocytes can present antigen to T cells. Indeed there is evidence against this notion. Thus, if antigen bound to B cells could trigger helper cell induction, removal of B cells from the inoculum of F_1 T cells used for activation should be a critical step for demonstrating restriction in helper function. Preliminary experiments suggest that this is not the case since restriction in helper function after activation in parental strain mice was no less evident when normal LN (which contain 30–40% B cells but few macrophages) rather than nylon-wool-passed T cells were used for activation (unpublished data). Macrophages (or related cells) are more likely candidates for antigen presentation in the present system, not only because of their well-accepted role in T-cell triggering, but also because they are highly radioresistant (23). The fact that the restriction in helper function could be overcome by the addition of PE cells (>80% macrophages) of the opposite parental strain (Table VI) supports this view. However, the precise identity of the cell(s) presenting antigen has yet to be established.

Whatever the nature of the cell presenting antigen to the T cell, it is clearly necessary to assume that despite the particulate nature of the antigen used (heterologous erythrocytes), the T cells were unable to recognize the antigen in terms of helper cell induction unless the antigen was first processed in a specific way in the irradiated environment. Thus, of the two putative T-cell subgroups in the F_1 T cell inoculum, only one apparently underwent clonal expansion when exposed to antigen in irradiated mice of one parental strain; the other subgroup failed to recognize the antigen and therefore remained in an unprimed (nonexpanded) state. Although this interpretation is in line with the generally accepted view that T-macrophage interactions are under MHC control, it is less easy to understand why, in the present system, such interactions were associated with apparent restrictions at the level of T-B collaboration.

One explanation for this paradox is that the data reflect restrictions acting at both the level of T-macrophage interactions and during T-B collaboration. Accordingly, one could argue that each subgroup of F_1 T cells is reactive to MHC-associated antigen presented first on host macrophages during helper cell induction in one of the two parental strain environments, and second, on the corresponding population of parental strain B cells during T-B collaboration. T-cell activation in one parental strain would thus induce clonal expansion of the subgroup of F_1 T cells able to collaborate only with B cells of that parental strain (or with F_1 B cells). The other T-cell subgroup would not be stimulated and would consequently give only a low primary response with its corresponding B-cell population, i.e. B cells of the opposite parental strain. The magnitude of this response would be equivalent to that of unprimed F_1 T cells.

The data, in toto, are consistent with this viewpoint. Nevertheless, it is clearly difficult to assess this interpretation without information on the genetic control of the restriction(s) observed. It is also necessary to consider another possibility; that the failure of F_1 T cells positively selected in one parent to collaborate with B cells of the opposite parent simply reflected a lack of appropriate macrophages during the stage of T-B collaboration. In other words, the cells failed to manifest their helper function because on subsequent transfer with B cells of the strain opposite to that used for activation, the macrophages presenting antigen to the helper cells were now different from those encountered during the initial activation. Although this possibility might seem unlikely since F_1 mice were always used for measurement of T-B collaboration, it needs to be excluded.

The following paper is addressed to this question and shows that the restriction(s) observed in the present paper cannot be explained in terms of a lack of appropriate macrophages during the stage of T-B collaboration. The data are interpreted as indicating that restrictions do exist at the level of both T helper cell induction and T-B collaboration, each restriction mapping to the *K*-end of the *H-2* complex.

Summary

Unprimed (CBA × C57BL/6)F_1 lymph node T cells were transferred with sheep erythrocytes (SRC) into heavily irradiated F_1 or parental strain mice and recovered from thoracic duct lymph or spleens of the recipients 5 days later. To study their helper function, the harvested F_1 T cells were transferred with antigen into irradiated F_1 mice plus B cells from either the two parental strains or from F_1 mice. F_1 T cells activated in F_1 mice gave high IgM and IgG anti-SRC responses with all three populations of B cells. By contrast, F_1 T cells activated in mice of one parental strain collaborated well with B cells of this strain, but poorly with B cells of the opposite strain. Active suppression was considered an unlikely explanation for this result since (*a*) good responses were found with F_1 B cells, and (*b*) addition experiments showed that the poor response with B cells of the opposite parental strain (which was equivalent to that produced by unprimed F_1 T cells) could be converted to a high response by a supplemental injection of F_1 T cells activated in F_1 mice. The phenomenon (*a*) was specific for the antigen used for activation (criss-cross experiments were performed with horse erythrocytes), (*b*) was reflected in levels of serum hemagglutinins as well as in numbers of splenic plaque-forming cells, (*c*) applied also to comparable activation of (DBA/2 × C57BL/6)F_1 T cells, and (*d*) could be prevented by activating F_1 T cells in mice of one parental strain in the presence of peritoneal exudate cells of the opposite parental strain.

The hypothesis was advanced that F_1 T cells contain two discrete subpopulations of antigen-reactive cells, each subject to restrictions acting at two different levels: (*a*) during T-macrophage interactions and (*b*) during T-B collaboration. It was suggested that when F_1 T cells are activated to antigen in a parental strain environment, radioresistant macrophages activate only one of the two subgroups of T cells, and this subgroup is able to collaborate with B cells of the strain used for activation (and with F_1 B cells) but not with B cells of the opposite parental strain. The other subgroup of T cells remains in an unprimed (nonactivated) state.

Stimulating discussion with D. B. Wilson, the technical assistance of Ms. L. Collins, and the skillful typing of Miss K. D. Nowell are gratefully acknowledged.

Received for publication 22 December 1977.

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RESTRICTED HELPER FUNCTION OF F_1 HYBRID T CELLS POSITIVELY SELECTED TO HETEROLOGOUS ERYTHROCYTES IN IRRADIATED PARENTAL STRAIN MICE

II. Evidence for Restrictions Affecting Helper Cell Induction and T-B Collaboration, Both Mapping to the *K*-End of the *H-2* Complex*

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The preceding paper demonstrated that F_1 hybrid T cells activated to heterologous erythrocytes for 5 days in irradiated mice of one parental strain cooperated well with B cells derived from either this strain or from F_1 mice, but not with B cells of the opposite parental strain. This restricted helper activity could not be accounted for in terms of active suppression. The data implied that F_1 T cells contain two discrete subpopulations of antigen-reactive cells, each reactive to antigen presented by radioresistant cells, probably macrophages, of one of the two parental strains. Why the mode of antigen presentation in the parental strain environment affected the capacity of the T cells to collaborate with B cells, however, was not clear. One explanation is that the poor collaboration observed with B cells of the opposite parental strain was simply the result of a failure to reactivate the T cells to antigen on subsequent transfer with B cells. This might occur if, during T-B collaboration, the macrophages presenting antigen to the T cells were of the wrong type, i.e. they did not share major histocompatibility complex (MHC)[1] determinants with the macrophages responsible for the initial activation of the T cells. Recent studies by Erb et al. (1) provide a precedent for this notion. These workers observed that F_1 T cells activated to antigen in vitro in the presence of parental strain macrophages failed to stimulate B cells of the opposite parent, though F_1 B cells gave good responses. Significantly, the inability to stimulate B cells of the opposite parental strain could be overcome by supplementing the B cells with macrophages sharing MHC determinants with the macrophages used for initial T-cell activation.

The present paper demonstrates that the addition of appropriate macrophages during T-B collaboration in vivo fails to abrogate the restriction in T helper function. It is concluded, therefore, that the system reflects restrictions imposed not only at the level of helper cell induction, but also during T-B collaboration. Studies with *H-2*-congenic and recombinant strains suggest that the restrictions at each of these two levels map to the *K*-end of the *H-2* complex.

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* Supported by grants AI-10961 and CA-15822 from the U. S. Public Health Service.

[1] *Abbreviations used in this paper:* BM, bone marrow; MHC, major histocompatibility complex; PE, peritoneal exudate; PFC, plaque-forming cells; SRC, sheep red blood cells; TDL, thoracic duct lymphocytes.

Materials and Methods

Mice. CBA Cum(CBA), C57BL/6 Cum(B6) and (CBA × B6)F_1 mice were obtained from Cumberland View Farms, Clinton, Tennessee. C57BL/10J (B10), B10.Br, DBA/2J, B10.D2, (B10 × B10.D2)F_1 and B10.A(5R) mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. B10.A(4R) mice were a gift of Dr. W. Elkins, University of Pennsylvania; these mice were bred at the University of Pennsylvania from lines provided by Dr. F. Bach of the University of Wisconsin at Madison.

Experimental Plan. The details of the experimental plan were reported in full in the preceding paper. Briefly, 5×10^7 nylon-wool-purified (>90% thy 1.2-positive) T cells from lymph nodes of unprimed (CBA × B6)F_1 mice were transferred intravenously with 0.5 ml of 25% sheep erythrocytes (SRC) into intermediate hosts which had received 800 rads the previous day. Thoracic duct fistulas were inserted in the cell recipients 5 days later, and thoracic duct lymphocytes (TDL) were collected overnight. These cells, >95% of which were thy 1.2-positive cells of donor origin, were transferred intravenously with B cells ($5\text{–}10 \times 10^6$ viable, anti-thy 1.2-serum-treated spleen or TDL from SRC-primed mice) and with 0.1 ml of 5% SRC into irradiated (750 rads) (CBA × B6)F_1 mice. Direct (IgM) and indirect (IgG) anti-SRC plaque-forming cells (PFC) were measured in the spleens of the recipients 7 days later. In some experiments, (DBA/2 × B6)F_1 T cells were used for activation. With these cells, T-B collaboration was measured by transferring the T cells and appropriate B cells into irradiated (DBA/2 × B6)F_1 mice.

Anti-Allotype Antiserum. Anti-Ig^b allotype was kindly provided by Dr. N. Klinman, University of Pennsylvania. The antiserum was made according to the technique of Herzenberg and Warner (2) by injecting BALB/c mice with anti-pertussis antibodies derived from B6 mice. Testing with the Ouchterlony technique showed that the antiserum was specific for serum from B6, B10, B10.Br, and (CBA × B6)F_1 mice, but not for serum from CBA, C3H, or BALB/c mice. The precise specificity of the antiserum was not determined. Antibodies directed against more than one IgG subclass were probably involved, since the antiserum enhanced a high proportion (60–80%) of the total anti-SRC PFC developed by a polyvalent rabbit anti-mouse Ig antiserum. The enhancing activity of the antiserum was specific for Ig^b allotypes in that PFC enhancement was observed with B6, B10, and B10.Br mice, but not with CBA or C3H mice.

PFC enhancement was studied by adding 1 drop of the antiserum (diluted 1:10) to the reaction mixture of spleen cells, SRC, and guinea pig serum as a source of complement (see preceding paper). PFC were then counted after transferring the mixture to Cunningham chambers. Numbers of PFC enhanced by the anti-allotype antiserum were calculated by subtracting numbers of direct (IgM) PFC obtained in the absence of the antiserum. Total indirect (IgG) PFC were estimated by subtracting numbers of direct PFC from the numbers of PFC obtained when rabbit anti-mouse Ig antiserum was added to the reaction mixture.

Bone Marrow (BM) Cells. BM cells were obtained from femurs and tibiae of 6- to 8-wk-old mice by flushing with balanced salt solution via a 26-gauge needle attached to a syringe. After counting, the cells were treated with anti-thy 1.2 serum (0.1 ml of undiluted antiserum/2×10^7 cells) and complement in a two-step procedure as described for spleen cells in the preceding paper.

Peritoneal Exudate Cells. Cells from the peritoneal cavity of mice given 1 ml of 4% thyoglycollate 4 days earlier were treated with anti-thy 1.2 serum and complement as for spleen cells. Over 80% of these cells had features of macrophages, i.e. they were large cells containing numerous granules.

Results

Failure to Explain Restricted Helper Function of Positively Selected F_1 T Cells as Due to Lack of Appropriate Macrophages during T-B Collaboration. As described in detail in the preceding paper, and as exemplified in Table I, unprimed (CBA × B6)F_1 T cells activated to SRC for 5 days in irradiated B6 mice (F_1 $T_{+(SRC\text{-}B6)}$) cooperated poorly with CBA B cells, but gave good responses with F_1 B cells. In contrast, F_1 T cells activated to SRC in irradiated F_1 mice (F_1 $T_{+(SRC\text{-}F_1)}$) stimulated both populations of B cells. Conceivably, this failure of F_1 $T_{+(SRC\text{-}B6)}$ cells to provide help for CBA B cells might reflect that the helper cells

TABLE I

*Helper Activity of TDL from Irradiated B6 Mice Given Unprimed (CBA × B6)F_1 T Cells Plus SRC 5 Days Previously: Effect of Supplementing Helper Cells with Macrophage-Containing Cells from (CBA × B6)F_1 Mice**

Cells transferred			Dose of SRC transferred with T and B cells	Anti-SRC PFC/spleen at 7 days in irradiated (CBA × B6)F_1 mice	
T cells‡ (CBA × B6)F_1	B cells (anti-thy 1.2 spl.)§	Macrophage-containing cells‖		IgM	IgG
F_1 $T_{\rightarrow(SRC\rightarrow B6)}$	(CBA × B6)F_1	–	0.1 ml of 5%	12,350 (1.13)¶	36,400 (1.14)
	CBA	–	0.1 ml of 5%	250 (1.57)	350 (1.18)
	CBA	–	0.1 ml of 50%	170 (1.39)	220 (1.20)
	CBA	F_1 PE cells	0.1 ml of 5%	750 (1.46)	310 (1.37)
	CBA	F_1 BM cells	0.1 ml of 5%	230 (1.36)	870 (1.45)
F_1 $T_{\rightarrow(SRC\rightarrow F_1)}$	(CBA × B6)F_1	–	0.1 ml of 5%	48,470 (1.12)	81,390 (1.14)
	CBA	–	0.1 ml of 5%	12,310 (1.04)	49,100 (1.18)
	CBA	F_1 PE cells	0.1 ml of 5%	7,620 (1.04)	26,630 (1.17)
	CBA	F_1 BM cells	0.1 ml of 5%	6,320 (1.22)	36,750 (1.18)

* Cell recipients were exposed to 800 rads 1 day before being injected intravenously with 5 × 10^7 unprimed nylon-wool-purified (CBA × B6)F_1 T cells (>90% thy 1.2-positive) plus 0.5 ml of 25% SRC.

‡ Recipients of F_1 T cells and SRC were cannulated at 5 days post-transfer, and the lymph-borne cells were collected overnight. The helper activity of the cells was measured by transferring 0.8 × 10^6 TDL (nearly all of which were shown by appropriate alloantisera to be T cells of donor F_1 origin) intravenously into irradiated (750 rads) (CBA × B6)F_1 mice plus B cells and 0.1 ml of 5% SRC.

§ Spleen cells from mice primed with SRC 2-4 mo previously and treated with anti-thy 1.2 serum plus complement before injection; 5 × 10^6 viable cells transferred.

‖ 4 × 10^6 viable cells transferred after treatment with anti-thy 1.2 antiserum plus complement. Cells were mixed with T cells, B cells, and SRC, and were injected intravenously.

¶ Geometric mean of data from 4 mice per group; number in parentheses refers to value by which mean is multiplied or divided to give upper and lower limits, respectively, of SE. Background values given by B cells transferred without T cells have been subtracted. These values (PFC/spleen) were: F_1 500(1.13) (IgM), 750(1.23) (IgG); CBA 150(1.30) (IgM), 140(1.22) (IgG); CBA + F_1 PE 90(1.46) (IgM), 190(1.37) (IgG); CBA + F_1 BM 490(1.40) (IgM), 180(1.74) (IgG). Values for T cells transferred without B cells were all <200 PFC/spleen. In the table values in parentheses used to derive SE were calculated before subtraction of background values.

were not reactivated during the stage of T-B collaboration. This could occur if the macrophages presenting the antigen at this stage did not share MHC determinants with the macrophages responsible for the initial activation of the T cells. In considering this possibility, it is essential to emphasize that T-B collaboration was measured in irradiated F_1 mice, i.e. in a situation in which the host macrophages were semi-syngeneic with respect to both the T cells and B cells. Nevertheless, it is possible that when the mixture of T cells, B cells, and SRC were transferred to the irradiated F_1 mice, the antigen was processed not by radioresistant host macrophages, but by the macrophages contaminating the B-cell source (anti-thy 1.2-treated spleen cells). These macrophages would not

TABLE II

Helper Activity of TDL from Irradiated B6 Mice Given Unprimed (CBA × B6)F_1 T Cells Plus SRC 5 Days Previously: Effect of Using CBA TDL as Source of B Cells[*]

T cells‡ (CBA × B6)F_1	B cells (anti-thy 1.2-treated)§	Anti-SRC PFC/spleen at 7 days in irradiated (CBA × B6)F_1 mice	
		IgM	IgG
F_1 $T_{+(SRC-B6)}$	CBA spleen	370 (1.12)‖	740 (1.47)
	CBA TDL	130 (1.53)	120 (1.58)
	B6 spleen	15,240 (1.24)	32,430 (1.17)
	(CBA × B6)F_1 spleen	14,080 (1.19)	47,020 (1.25)
F_1 $T_{+(SRC-F_1)}$	CBA spleen	13,630 (1.09)	72,050 (1.07)
	CBA TDL	12,740 (1.04)	58,080 (1.06)
	B6 spleen	16,710 (1.14)	38,470 (1.09)
	(CBA × B6)F_1 spleen	45,810 (1.16)	145,080 (1.18)

*, ‡ As for Table I.

§ As for Table I. TDL were from first overnight collection of lymph; both spleen and TDL were transferred in a dose of 5 × 10^6 viable cells after treatment with anti-thy 1.2 serum plus complement; B6 spleen cells were transferred in a dose of 8 × 10^6 viable cells.

‖ As for footnote ¶ of Table I. Background values for B cells transferred without T cells have been subtracted. These values (PFC/spleen) were: CBA spleen < 100 (IgM), < 100 (IgG); CBA TDL < 100 (IgM), 260(1.35) (IgG); B6 spleen 660(1.17) (IgM), 1,770(1.51) (IgG); F_1 spleen 190(1.64) (IgM), 640(1.86) (IgG). Numbers of PFC for T cells transferred without B cells were all <100 PFC /spleen.

share MHC determinants with the macrophages used for initial T-cell activation, and they would therefore fail to reactivate the T cells.

Four approaches were used to investigate this possibility. The first was simply to increase the dose of antigen adoptively transferred with the T and B cells, thereby making it less likely that macrophages contaminating the B-cell source could preempt antigen-processing by host macrophages. As shown in Table I, increasing the challenge dose 10-fold to 0.1 ml of 50% SRC failed to overcome the poor response obtained with CBA splenic B cells.

The second approach was to supplement the transfer mixture of T and B cells with a cell source enriched for macrophages which shared MHC determinants with the strain used for T-cell activation. As shown in Table I, addition of 4 × 10^6 anti-thy 1.2-treated peritoneal exudate (PE) cells (>80% macrophages) or BM cells (a source of stem cells and macrophage precursors) obtained from F_1 mice did not influence the poor helper function of F_1 $T_{+(SRC-B6)}$ cells for B cells prepared from CBA spleen. Similar results were observed when the added PE or BM cells were taken from B6 rather than from F_1 mice (data not shown).

The third approach was to use B cells which were depleted of macrophages. TDL, i.e. cells containing virtually no macrophages (3), were used for this purpose. It can be seen from Table II that the helper function of F_1 $T_{+(SRC-B6)}$ cells for CBA B cells was very low, irrespective of whether anti-thy 1.2-treated TDL or spleen cells were used as a source of B cells. Both B-cell populations gave good responses with F_1 $T_{+(SRC-F_1)}$ cells.

The final approach was to measure T-B collaboration in F_1 mice which had been irradiated and injected intravenously with F_1 BM cells (3 × 10^6 anti-thy

TABLE III

*Helper Activity of TDL from Irradiated B6 Mice Given Unprimed (CBA × B6)F_1 T Cells Plus SRC 5 Days Previously: Effect of Measuring T-B Collaboration in Irradiated (CBA × B6)F_1 Mice Reconstituted with F_1 BM 6 Days Earlier**

T cells‡	B cells§ (anti-thy 1.2-treated)	Pretreatment of F_1 recipients of T and B cells‖	Anti-SRC PFC/spleen at 7 days in irradiated (CBA × B6)F_1 mice	
			IgM	IgG
F_1 $T_{+(SRC-B6)}$	B6 spleen	–	32,970 (1.13)¶	59,070 (1.17)
	(CBA × B6)F_1 spleen	–	43,600 (1.21)	85,740 (1.23)
	CBA spleen	–	780 (1.46)	1,620 (1.11)
	CBA spleen	F_1 BM 6 days before	260 (1.20)	1,340 (1.21)
	CBA TDL	–	260 (1.20)	2,320 (1.31)
	CBA TDL	F_1 BM 6 days before	680 (1.95)	720 (1.85)
F_1 $T_{+(SRC-F_1)}$	B6 spleen	–	9,280 (1.35)	18,010 (1.38)
	(CBA × B6)F_1 spleen	–	74,700 (1.04)	180,360 (1.00)
	CBA spleen	–	12,350 (1.41)	51,330 (1.21)
	CBA spleen	F_1 BM 6 days before	2,010 (1.25)	11,480 (1.41)
	CBA TDL	–	4,510 (1.17)	28,470 (1.16)
	CBA TDL	F_1 BM 6 days before	3,960 (1.24)	10,000 (1.56)

*, ‡ As for Table I.

§ As for Table I. TDL were from first overnight collection of lymph; both spleen and TDL were transferred in a dose of 5 × 10^6 viable cells (8 × 10^6 for B6 spleen) after treatment with anti-thy 1.2 serum plus complement.

‖ Mice were given 850 rads followed by intravenous injection of 3 × 10^6 anti-thy 1.2-treated F_1 BM cells 4 h later. These mice were then used as recipients of T and B cells 6 days later. Mice not pretreated received 750 rads 18 h before T- and B-cell transfer.

¶ As for Table I. Background values of B cells transferred without T cells have been subtracted. These values (PFC/spleen) were: B6 spleen 270(1.78) (IgM), 1,140(1.69) (IgG); F_1 spleen 390(1.31) (IgM), 1,340(1.35) (IgG); CBA spleen 160(1.31) (IgM), 150(1.50) (IgG); CBA spleen + F_1 BM 130(1.71) (IgM), 210(1.19) (IgG); CBA TDL <100 (IgM), <100 (IgG); CBA TDL + F_1 BM 870(1.43) (IgM), 1,330(1.62) (IgG).

1.2-treated cells) 6 days previously. At the time of transferring the T and B cells into these mice, the spleens of the recipients were of near normal size and contained large numbers (~5 × 10^7/spleen) of newly differentiated hemopoietic cells, including cells with the morphology of macrophages. This approach was designed to answer the objection to the first approach that mature macrophages injected intravenously might not home to the spleen. As shown in Table III, the poor help provided by F_1 $T_{+(SRC-B6)}$ cells for CBA B cells (from spleen) was not improved when the irradiated F_1 recipients of the T and B cells had been reconstituted with syngeneic BM cells 6 days previously. Similar results were obtained when B6 BM rather than F_1 BM was used to reconstitute the irradiated F_1 mice (data not shown). A more stringent test was to combine the third and fourth approaches and transfer the F_1 $T_{+(SRC-B6)}$ cells into the F_1-BM-reconstituted F_1 mice along with macrophage-depleted cells as a source of B cells, i.e.

TABLE IV

*Helper Activity of TDL from Irradiated CBA and B6 Mice Given (CBA × B6)F_1 T Cells Plus SRC 5 Days Previously: Response with B10.Br and B10 B Cells**

T cells‡ (CBA × B6)F_1	B cells (*H-2* region, Ig allotype)§		Anti-SRC PFC/spleen at 7 days in irradiated F_1 mice			
			IgM	IgG	Ig^b‖	Ig^b/IgG
F_1 $T_{+(SRC-CBA)}$	CBA	(H-2^k, Ig^a)	12,330 (1.18)¶	49,800 (1.19)	0	0.00
	B10.Br	(H-2^k, Ig^b)	41,510 (1.30)	113,730 (1.40)	86,620 (1.18)	0.76
	B10	(H-2^b, Ig^b)	1,040 (1.17)	2,120 (1.26)	1,440 (1.16)	0.68
	(CBA × B6)F_1	(H-2^k, × H-2^b, Ig^a × Ig^b)	30,640 (1.17)	99,950 (1.04)	43,980 (1.13)	0.44
F_1 $T_{+(SRC-B6)}$	CBA	(H-2^k, Ig^a)	1,140 (1.31)	340 (1.94)	0	0.00
	B10.Br	(H-2^k, Ig^b)	480 (1.27)	1,080 (1.39)	830 (1.38)	0.77
	B10	(H-2^b, Ig^b)	27,080 (1.13)	62,700 (1.19)	48,130 (1.12)	0.77
	(CBA × B6)F_1	(H-2^k, × H-2^b, Ig^a × Ig^b)	14,130 (1.28)	46,950 (1.24)	15,490 (1.13)	0.33

*, ‡, § As for Table I; 5 × 10^6 B cells (anti-thy 1.2-treated spleen) were transferred (8 × 10^6 for B10 B cells).

‖ Number of PFC developed with BALB/c anti-B6-Ig (anti-Ig^b) antiallotype antiserum (see Materials and Methods).

¶ As for Table I. Background values for B cells transferred without B cells have been subtracted. These values (PFC/spleen) were: CBA 430(1.17) (IgM), <100 (IgG), 0 (Ig^b); B10.Br 710(1.27) (IgM), 280(1.86) (IgG), 230(1.67) (Ig^b); B10 340(1.21) (IgM), 660(2.40) (IgG), 500(1.29) (Ig^b); F_1 510(1.52) (IgM), 360(1.35) (IgG), 210(1.61) (Ig^b).

anti-thy 1.2-treated TDL. Again, this failed to overcome the poor helper function of F_1 $T_{+(SRC-B6)}$ cells for CBA B cells. It may be noted that responses observed with the control population of T cells (F_1 $T_{+(SRC-F_1)}$) were appreciably lower when BM-reconstituted F_1 mice were used to measure T-B collaboration, possibly because such mice have less available "space" for generating the immune response (4).

Role of the H-2 Complex in the Restricted Helper Function of Positively Selected F_1 T Cells. The data shown in Table IV indicate that the restricted helper function of (CBA × B6)F_1 T cells activated to SRC in parental strain mice is linked to the *H-2* complex, but not to immunoglobulin allotype. With respect to the *H-2* complex, it is evident that F_1 $T_{+(SRC-CBA)}$ cells collaborated well with CBA (H-2^k) B cells and B10.Br (H-2^k) B cells, but poorly with B10 (H-2^b) B cells (B10.Br and B10 differ only with respect to the *H-2* complex; B10.Br and CBA have different genetic backgrounds, as do B6 (H-2^b) and B10). Conversely, F_1 $T_{+(SRC-B6)}$ cells stimulated B10 B cells, but did not cooperate well with either CBA or B10.Br B cells.

The fact that CBA and B10.Br have different Ig allotypes (see Table IV) would seem to rule out the possibility that the restriction in helper function observed with CBA and B6 (or B10) B cells reflected the differing allotypes on these cells (Ig^a and Ig^b, respectively). It can be seen from Table IV that the restriction in helper function as measured by the numbers of IgM and IgG PFC also applied to numbers of PFC enhanced by a polyvalent anti-Ig^b anti-allotype serum. It is to be noted that both populations of T cells stimulated (CBA × B6)F_1 B cells to produce Ig^b PFC. As expected, the proportion of Ig^b PFC (relative to the IgG PFC enhanced by the polyvalent anti-mouse Ig antiserum) was appreciably less with allotype-heterozygous (CBA × B6)F_1 B cells (~40%) than with Ig^b-homozygous B cells (~75%).

The data shown in Table V indicate that the restricted helper function of positively selected F_1 T cells is controlled by genes mapping in the *K*-end of the *H-2* complex. In this experiment, (CBA × B6)F_1 T cells were activated to SRC

TABLE V

*Helper Function of TDL from Irradiated B10, B10.Br, and B10.A(4R) Mice Injected with Unprimed (CBA × B6)F$_1$ T Cells Plus SRC 5 Days Previously: Responses with B10, B10.Br, and B10.A(4R) B Cells**

T cells‡ (CBA × B6)F$_1$	B cells§ (anti-thy 1.2 spleen)	*H-2* region of B cells‖					Anti-SRC PFC/spleen at 7 days in irradiated (CBA × B6)F$_1$ mice	
		K	*I-A*	*I-B*	----	*D*	IgM	IgG
F$_1$ T$_{+(\text{SRC-B10})}$	B10	*b*	*b*	*b*	----	*b*	33,360 (1.06)¶	165,640 (1.09)
	B10.Br	*k*	*k*	*k*	----	*k*	2,200 (1.33)	7,200 (1.30)
	B10.A(4R)	*k*	*k* |	*b*	----	*b*	630 (1.43)	2,590 (1.48)
F$_1$ T$_{+(\text{SRC-B10.Br})}$	B10	*b*	*b*	*b*	----	*b*	1,420 (1.77)	4,410 (1.46)
	B10.Br	*k*	*k*	*k*	----	*k*	44,510 (1.03)	146,420 (1.10)
	B10.A(4R)	*k*	*k* |	*b*	----	*b*	25,410 (1.08)	134,020 (1.25)
F$_1$ T$_{+(\text{SRC-B10.A(4R)})}$	B10	*b*	*b*	*b*	----	*b*	1,150 (1.27)	4,330 (1.13)
	B10.Br	*k*	*k*	*k*	----	*k*	22,950 (1.01)	77,030 (1.16)
	B10.A(4R)	*k*	*k* |	*b*	----	*b*	15,590 (1.16)	69,320 (1.13)

*, ‡ As for Table I.

§ As for Table I. B cells transferred in a dose of 5 × 10^6 viable cells for B10.Br spleen and 8 × 10^6 cells for B10 and B10.A(4R) spleen.

‖ Verticle lines denote the presumed position of the cross-over in B10.A(4R).

¶ As for Table I. Background values for B cells alone have been subtracted. These values (PFC/spleen) were: B10 330(1.46) (IgM), 850(1.42) (IgG); B10.Br 1,270(1.67) (IgM), 1,200(1.17) (IgG); B10.A(4R) 220(1.25) (IgM), 1,740(1.51) (IgG).

in irradiated B10, B10.Br, or B10.A(4R) (K^k $I\text{-}A^k$ $I\text{-}B^b$ . . . D^b) mice, and then tested for their capacity to stimulate B cells derived from these three strains. Two points arise from this experiment. First, the helper function of F$_1$ T cells activated in B10.A(4R) mice resembled that of cells activated in B10.Br mice, in that both T-cell populations gave good responses with B10.Br B cells, but not with B10 B cells; the reverse applied to T cells activated in B10 mice. This suggests that during positive selection in the irradiated intermediate hosts, T-cell recognition of gene products mapping to the left of the *I-B* subregion determined which subgroup of F$_1$ T cells was to be activated. Second, it can be seen that T-cell triggering of B10.A(4R) B cells resembled that of B10.Br B cells rather than B10 B cells. Thus, both B10.A(4R) B cells and B10.Br B cells were triggered by F$_1$ T cells activated in either B10.A(4R) mice or B10.Br mice; B10 B cells were stimulated only by F$_1$ T cells activated in B10 mice. This implies that, like T-cell activation, collaboration with B cells depends upon T-cell recognition of *K* and/or *I-A* subregion determinants.

The data shown in Table VI represent a similar study with (DBA/2 × B6)F$_1$ T cells. When these cells were activated to SRC in irradiated B6 mice (F$_1$ T$_{+(\text{SRC-B6})}$ cells), poor responses were found with both DBA/2 ($H\text{-}2^d$) and B10.D2 ($H\text{-}2^d$ on the B10 background) B cells, whereas good cooperation was seen with B10 ($H\text{-}2^b$), B10.A(5R) (K^b $I\text{-}A^b$ $I\text{-}B^b$ $I\text{-}J^k$ $I\text{-}E^k$ $I\text{-}C^d$ D^d), and (B10.D2 × B10)F$_1$ B cells (T-B collaboration was measured in irradiated (DBA/2 × B6)F$_1$ mice). Opposite results were observed with F$_1$ T$_{+(\text{SRC-DBA/2})}$ cells, i.e. poor cooperation was seen

TABLE VI

*Helper Activity of TDL from Irradiated B6 and DBA/2 Mice Given Unprimed (DBA/2 × B6)F_1 T Cells Plus SRC 5 Days Previously: Responses with B10.A(5R) B Cells**

T cells‡ (DBA/2 × B6)F_1	B cells§ (anti-thy-1.2-spleen)	*H-2* region of B cells‖							Anti-SRC PFC/spleen at 7 days in irradiated (DBA/2 × B6)F_1 mice	
		K	*I-A*	*I-B*	*I-J*	*I-E*	*I-C*	*D*	IgM	IgG
F_1 $T_{+(SRC-B6)}$	DBA/2	*d*	——→					*d*	430 (1.28)¶	1,250 (1.45)
	B10.D2	*d*	——→					*d*	750 (1.25)	660 (1.37)
	B10.A(5R)	*b*	*b*	*b* |	*k*	*k* |	*d*	*d*	30,190 (1.27)	87,580 (1.18)
	B10	*b*	——→					*b*	7,190 (1.08)	20,870 (1.14)
	(B10.D2 × B10)F_1	*d/b*	——→					*d/b*	14,700 (1.11)	35,040 (1.22)
F_1 $T_{+(SRC-DBA/2)}$	DBA/2	*d*	——→					*d*	13,010 (1.40)	55,540 (1.11)
	B10.D2	*d*	——→					*d*	49,750 (1.15)	88,420 (1.08)
	B10.A(5R)	*b*	*b*	*b* |	*k*	*k* |	*d*	*d*	920 (1.28)	4,760 (1.24)
	B10	*b*	——→					*b*	190 (1.25)	200 (1.28)
	(B10.D2 × B10)F_1	*d/b*	——→					*d/b*	24,510 (1.13)	55,600 (1.14)

*, ‡ As for Table I.

§ As for Table I. All B cells transferred in a dose of 5 × 10^6 viable cells.

‖ Vertical lines indicate the presumed position of the crossovers in B10.A(5R).

¶ As for Table I. Background values for B cells transferred alone have been subtracted. These values (PFC/spleen) were: DBA/2 170(1.19) (IgM), 220(1.65) (IgG); B10.D2 170(1.39) (IgM), 180(1.61) (IgG); B10.A(5R) 950(1.48) (IgM), 1,940 (IgG); B10 100(1.29) (IgM), < 100 (IgG); (B10.D2 × B10)F_1 550(1.21) (IgM), 180 (1.84) (IgG).

with both B10 and B10.A(5R) B cells. These findings with B10.A(5R) B cells suggest that, as for the (H-2^k × H-2^b)F_1 combination (Table V), activation of (H-2^d × H-2^b)F_1 T cells in a parental strain environment generated helper cells which collaborated specifically with B cells sharing the left-hand region of the *H-2* complex with the strain used for activation. Attempts were not made to study the effect of T-cell activation in B10.A(5R) mice, since the H-2^k determinants expressed by the *I-J* and *I-E* subregions of these mice might in theory have induced allosensitization. D2.GD (K^d I-A^d I-B^b . . . D^b) mice would have been suitable for this purpose, but were not available.

On the Stimulation of F_1 B Cells by F_1 T Cells Activated to Antigen in Parental Strain Mice. Data presented in the preceding paper demonstrated that (CBA × B6)F_1 T cells positively selected to SRC in irradiated parental strain mice invariably gave good responses with F_1 B cells. Nevertheless, these responses tended to be lower than the responses observed when F_1 B cells were transferred with F_1 T cells activated in irradiated F_1 mice (F_1 $T_{+(SRC-F_1)}$). Conversely, parental strain B cells were triggered more effectively by F_1 T cells activated in irradiated mice of this parental strain than by F_1 $T_{+(SRC-F_1)}$ cells. These reciprocal differences, though not dramatic, were seen in 10 of 11 experiments, and they applied to both IgM and IgG PFC (see Table III of this paper, and Tables IV, V, and Figs. 1, 2 of preceding paper); the differences were reflected in titers of serum hemagglutinins as well as in numbers of splenic PFC measured at 5, 7, and 9 days post-transfer. The results of five experiments are tabulated in Table VII. In these experiments the helper functions of F_1 $T_{+(SRC-F_1)}$ cells and F_1 $T_{+(SRC-B6)}$ cells transferred at a dose of 0.8 × 10^6 cells were compared for their capacity to stimulate F_1 B cells and B6 B cells. For simplicity, the results are expressed in terms of the ratios of the responses obtained. In the case of F_1 B cells, it can be seen that F_1 $T_{+(SRC-F_1)}$ cells were more effective as helper cells than were F_1 $T_{+(SRC-B6)}$ cells. The ratio of PFC numbers produced by

TABLE VII

Relative Helper Function of (CBA × B6)F_1 T Cells Positively Selected to SRC in Irradiated F_1 or B6 Mice: Preferential Helper Function for B Cells Syngeneic with the Strain Used for T-Cell Activation

Ratio*	Number of T cells transferred with constant number of B cells	Type of PFC	Anti-SRC PFC with F_1 $T_{+(SRC-F_1)}$ / Anti-SRC PFC with F_1 $T_{+(SRC-B6)}$					
			Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Mean
1. Ratio with F_1 B cells	0.8 × 10^6	IgM	1.72	5.49	5.33	3.25	5.09	4.18
	0.8 × 10^6	IgG	2.10	2.59	2.53	3.09	2.89	2.64
2. Ratio with B6 B cells	0.8 × 10^6	IgM	0.28	0.24	0.24	1.10	0.68	0.51
	0.8 × 10^6	IgG	0.31	0.30	0.30	1.19	0.45	0.51
Ratio 1 ÷ Ratio 2		IgM	6.14	22.88	22.21	2.95	7.49	8.20
		IgG	6.77	8.63	8.43	2.60	6.42	5.18

* The data are expressed in terms of the mean number of PFC/spleen (geometric mean of four mice per group) measured at 7 days post-transfer. B cells (from spleen) transferred in a dose of 5 × 10^6 viable cells for F_1 B cells and 8 × 10^6 for B6 B cells.

these two T-cell populations was >1 (mean: 4.18 [IgM], 2.64 [IgG]). By contrast, with B6 cells the ratios were <1 in 4 of 5 experiments (mean: 0.51 [IgM], 0.51 [IgG]). For convenience these two ratios are compared in Table VII by dividing the first ratio by the second ratio. It is evident that this master ratio was considerably greater than unity in all five experiments.

It should be emphasized that these ratios were calculated from experiments in which a comparatively low dose of helper T cells (0.8 × 10^6) was used. In two other experiments, one of which is shown in Table VIII, the helper cells were transferred at both a high dose (2.4 × 10^6 cells) and a low dose (0.6 × 10^6). Both experiments gave comparable results. In the case of responses with F_1 B cells, both T-cell populations gave comparable responses when transferred in the higher dose, i.e. the ratio with F_1 B cells was now close to unity. A similar result was observed with B6 B cells, although with these cells a value of unity for the ratio, though approached, was not reached.

Discussion

A variety of approaches failed to show that the restricted helper function of F_1 T cells positively selected to SRC in irradiated parental strain mice reflects a lack of appropriate macrophages during T-B collaboration in vivo. Analogous findings have recently been reported by Swierkosz et al. (5) using an in vitro system. One is thus left with the possibility that the present data reflect restrictions acting not only at the level of helper cell induction, but also during T-B collaboration. With respect to T-B interaction, studies with congenic-resistant strains and recombinant mice suggested that for both (CBA × B6)F_1 T cells (Table V) and (DBA/2 × B6)F_1 T cells (Table VI), activation to antigen in irradiated mice of one parental strain selected for a population of helper cells

Table VIII

*Relative Helper Function of (CBA × B6)F_1 T Cells Positively Selected to SRC in Irradiated F_1 or B6 Mice: Preferential Helper Function for B Cells Syngeneic with the Strain Used for Activation Reduced or Abolished by Using High Dose of Helper T Cells**

Ratio	Number of T cells transferred with constant number of B cells‡	Type of PFC	Anti-SRC PFC with F_1 $T_{+(SRC-F_1)}$	/	Anti-SRC PFC with F_1 $T_{+(SRC-B6)}$	Ratio
1. Ratio with F_1 B cells	0.6 × 10^6	IgM	28,080 (1.15)§	/	6,330 (1.20)	4.44
	2.4 × 10^6	IgM	37,360 (1.12)	/	36,710 (1.09)	1.02
	0.6 × 10^6	IgG	97,450 (1.12)	/	34,940 (1.19)	2.79
	2.4 × 10^6	IgG	126,120 (1.12)	/	124,760 (1.07)	1.01
2. Ratio with B6 B cells	0.6 × 10^6	IgM	5,570 (1.21)	/	9,580 (1.27)	0.58
	2.4 × 10^6	IgM	22,040 (1.19)	/	22,730 (1.14)	0.97
	0.6 × 10^6	IgG	17,240 (1.37)	/	39,830 (1.14)	0.43
	2.4 × 10^6	IgG	58,030 (1.10)	/	79,490 (1.10)	0.73
Ratio 1 ÷ Ratio 2	0.6 × 10^6	IgM				7.66
	2.4 × 10^6	IgM				1.05
	0.6 × 10^6	IgG				6.49
	2.4 × 10^6	IgG				1.38

*,‡ As for Table I. B cells (from spleen) were transferred in a dose of 5 × 10^6 viable cells for F_1 B cells and 8 × 10^6 for B6 B cells.

§ As for footnote ¶ of Table I. PFC numbers for B cells transferred alone have been subtracted. These values were all < 1,000 PFC/spleen. PFC numbers for T cells transferred without B cells were < 200 PFC/spleen.

which collaborated only with B cells sharing the *K*-end of the *H-2* complex with the strain used for T-cell activation. Experiments with (CBA × B6)F_1 ($H\text{-}2^k$ × $H\text{-}2^b$) T cells and B10.A(4R) (K^k $I\text{-}A^k$ $I\text{-}B^b$ . . . D^b) B cells, indicated that the determinants controlling collaboration mapped to the left of the *I-B* subregion, i.e. in the *K* or *I-A* subregions. The determinants controlling T-cell activation (presumably a reflection of T-macrophage interactions, see preceding paper) also appeared to map to this region since (CBA × B6)F_1 T cells collaborated well with both B10.Br and B10.A(4R) B cells, but not with B10 B cells, irrespective of whether the T cells were activated in irradiated B10.Br or in B10.A(4R) mice (Table V).

Before assessing the significance of these findings it is important to consider recent evidence which suggests that, as with F_1 T cells, the response of parental strain T cells to SRC in vivo is subject to *H-2* gene control at the level of both T-macrophage interactions and T-B collaboration. This evidence was derived from experiments with T cells which had been depleted of specific alloreactive lymphocytes by an acute procedure, i.e. recirculation from blood to lymph through irradiated *H-2*-incompatible mice. First, T cells exposed to antigen in *H-2*-different hosts appeared to be unable to see the antigen in terms of helper cell induction. Thus, parental strain T cells transferred to irradiated mice in the presence of SRC underwent positive selection to the antigen (i.e., were induced to become activated helper cells) only when the cell recipients shared *H-2* determinants with the donor T cells (excellent selection occurred in semiallogeneic [F_1] mice).[2] Second, parental strain T cells failed to stimulate anti-SRC responses by allogeneic B cells unless the T and B cells shared *H-2* determinants, in particular *K/I-A* subregion determinants; effective collaboration occurred with F_1 B cells (6, and unpublished observations).

Two predictions follow from these data. Both center on the phenomenon of negative selection – a process occurring within 1-2 days of exposure to antigen which leads to a transient disappearance of specific antigen-reactive lymphocytes from the circulation, e.g. TDL. During negative selection, lymphocytes reactive to the injected antigen become temporarily sequestered in regions such as the spleen before reentering the circulation (7). The first prediction is that if negative selection always precedes positive selection (which is very likely), the failure to observe T-helper cell induction in irradiated *H-2*-different mice (*vide supra*) should be paralleled by an equivalent failure to observe negative selection. This has been verified by transferring purified parental-strain T cells with SRC into irradiated mice, and collecting the donor-derived cells from thoracic duct lymph of the recipients 1-2 days later. These T cells were selectively depleted of helper reactivity for the injected antigen but, significantly, only when the donor and host shared *H-2* determinants (*I*-region compatibility was sufficient, and effective selection occurred in semiallogeneic hosts).[2,3]

The second prediction, which follows from the first, is that the restriction in the helper function of F_1 T cells transferred with antigen to irradiated parental strain mice would change reciprocally depending upon whether the cells were harvested from the recipients during the stage of negative selection or during positive selection. Thus, if only one of the two putative subpopulations of F_1 T cells responded to the injected antigen, this subgroup would initially be withdrawn from the circulation to the lymphoid tissues, whereas the other population would ignore the antigen and continue to recirculate. If so, then by analogy with the present data on positive selection of F_1 T cells, F_1 cells taken from the lymph during the stage of negative selection should not collaborate with B cells derived from the strain used for activation, but should act as unprimed helper cells for B cells of the opposite parent and for F_1 B cells. Recent studies have verified this prediction.[4]

The above evidence on the in vivo response to SRC is in line with the observations of other workers indicating that T cells from homozygous mice are

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[2] J. Sprent. 1977. *H-2* gene control of homozygous T helper cell induction in vivo. Manuscript in preparation.

[3] These data apply to help provided for B cells syngeneic with the donor T cells. It might be suggested that selection to antigen would occur in *H-2*-different hosts if help were examined for B cells syngeneic with the allogeneic host. In this respect it should be emphasized that under no circumstances (with the exception of raising T cells in a chimeric environment) has the author observed collaboration between *H-2*-different T and B cells.

[4] J. Sprent. 1977. Restricted helper function of F_1 hybrid T cells revealed by negative selection through irradiated parental strain mice. Manuscript in preparation.

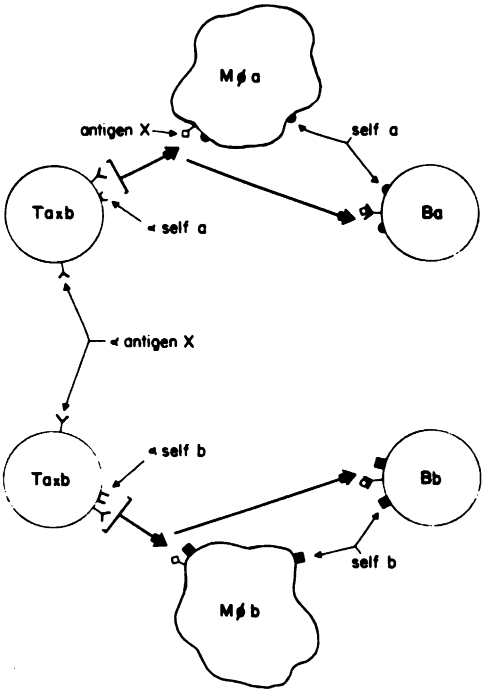

FIG. 1. $(a \times b)F_1$ T cells are envisaged as consisting of two discrete subgroups, each reactive to self-*H-2*-associated antigen presented by macrophages and also by specific B cells of one of the two parental strains. Restrictive interactions at these two levels are portrayed in terms of dual recognition, each T-cell subgroup expressing clonally distributed anti-self receptors for *H-2* (?*I-A*) determinants on the corresponding parental strain population of macrophages and B cells. In addition, both T-cell subgroups express identical receptors for antigen X; specific B cells carry analogous receptors for antigen X. Via both sets of receptors, T cells recognize *H-2*-associated antigen on macrophages and, after activation, trigger B cells which bear the corresponding *H-2* determinants and, in addition, carry antigen X in their Ig receptors. Each subgroup of T cells is reactive to F_1 macrophage-associated antigen and can help F_1 B cells.

subject to *H-2* gene control both at the level of T-macrophage interactions (8–11) and during T-B collaboration in vivo (12–14). At each of these two levels, the relevant genes mapped in the *I-A* subregion of the *H-2* complex. The fact that, at least with one strain combination, the restrictions observed in the present system both mapped to the left of the *I-B* subregion is clearly consistent with these data, although further work will be necessary to map the restrictions precisely. If the restrictions observed with F_1 T cells do map to the *I-A* subregion, one is led to the intriguing conclusion that F_1 T cells behave functionally as a mixture of (mutually tolerant) T cells derived from the two homozygous parental strains.

A question of obvious importance is whether the restrictions affecting T-macrophage interactions and T-B collaboration are controlled by the same gene (or set of genes) or, alternatively, by two closely linked genes. Although no evidence is available which bears directly on this point, it is conceptually simpler to think in terms of one gene. According to this view, both macrophages and specifically-reactive B cells present MHC-associated antigen to T cells in an identical or similar fashion. F_1 mice contain two subgroups of T cells, each of which is reactive to MHC-associated antigen presented by macrophages or specific B cells of one of the two parental strains. Thus, as illustrated in Fig. 1, positive selection of $(a \times b)F_1$ T cells via macrophage-associated antigen in irradiated parental strain *a* induces clonal expansion of the subgroup of T cells destined to help strain *a* B cells (and F_1 B cells). The subgroup of T cells reactive to strain *b* B cells is not stimulated and remains in an unprimed (nonexpanded) state. This subgroup is activated in strain *b* mice, and both subgroups are stimulated in the F_1 environment.

Fig. 1 portrays these restrictive interactions in terms of dual recognition. According to this viewpoint, each of the two subgroups of $(a \times b)F_1$ T cells express identical receptors for conventional antigen X. In addition, the cells carry another set of receptors with specificity for self *H-2* (presumably *I-A*) determinants of either strain *a* or strain *b*; these anti-self receptors are clonally distributed, or if alleleic, are allelically excluded (see 10, 13, 15, and 16 for other models of dual recognition). In the case of the F_1 T cells reactive to self $H\text{-}2^a$ determinants, these cells are triggered by a complex of $H\text{-}2^a$-X presented by (or possibly secreted by; (17) macrophages of strain *a*. The activated helper cells then stimulate B cells which express self $H\text{-}2^a$ determinants and, in addition, carry antigen X in their Ig receptors. Alternatively, the activated T cell could bind free antigen and focus it onto the appropriate B cell.

An analogous model could be constructed on the basis of altered self recognition (15). Here, one must postulate that altered self determinants are created on the surface of B cells as well as on macrophages; at the B-cell level, the *H-2* determinants on the cell would presumably have to be modified by antigen bound to the cell via Ig receptors.

Further genetic mapping studies and experiments with other antigens will be required before the above models can be evaluated critically. It will also be necessary to accommodate the conflicting evidence that T cells release factors which stimulate B cells across *H-2* barriers (18, 19), and that according to some groups (20–22), although not others (23), T-B collaboration measured in vitro proceeds across MHC barriers.

Finally, comment should be made on the puzzling finding that F_1 T cells cooperated preferentially with B cells syngeneic with the strain used for activation (Table VII). Thus, F_1 T cells activated to antigen in parental strain mice gave relatively better responses with B cells of that parental strain than with F_1 B cells; conversely, F_1 T cells activated in F_1 mice stimulated F_1 B cells more effectively than parental strain B cells. Significantly, these preferences were observed with small numbers of T cells ($\leq 0.8 \times 10^6$) but not with higher doses (Table VIII). This explains the help provided for parental strain B cells by F_1 T cells activated in F_1 mice. Here it can be argued that only one-half of the T cells generated in an F_1 environment provide help for one of the parental B-cell populations. Increasing the dose of helper cells should therefore give maximal help. The situation with the response of F_1 B cells is more complex. A priori, the phenomenon might reflect (*a*) a mild degree of suppression, (*b*) allelic exclusion on F_1 B cells of the *H-2* determinants controlling collaboration, (*c*) co-dominant expression of *H-2* determinants which, by competition, inhibit collaborative interactions with one of the two subgroups of F_1 T cells, or (*d*) the existence of a third subgroup of F_1 T cells which has specificity for hybrid-specific determinants on F_1 B cells (24). The fact that the phenomenon was not observed with higher doses of helper cells is against possibilities (*a*) and (*b*), unless one argues that for (*a*), F_1 B cells mediate the suppression, or that for (*b*), increasing help provided for one subgroup of B cells eventually leads indirectly to stimulation of the other subgroup. Various objections can be raised against the third possibility, not the least of which is the unsubstantiated corollary that T-B collaboration should be generally less efficient in F_1 mice than in homozygous mice. The fourth possibility is difficult to assess without further information.

Summary

Studies with *H-2*-congenic and recombinant strains showed that when F_1 hybrid T cells were activated to sheep erythrocytes in irradiated mice of parental strain or related strain, a population of helper cells was generated which collaborated only with B cells sharing the *K*-end of the *H-2* complex with the strain used for activation. No evidence was found that the restriction in helper function (*a*) reflected a deficiency of appropriate macrophages during T-B collaboration, or (*b*) was influenced by the Ig allotype of the B cells. It was concluded that the results signified restrictions acting at both the level of helper cell induction (presumed to be a reflection of T-macrophage interactions in the irradiated intermediate hosts) and during T-B collaboration. With (CBA × C57BL/6)F_1 T cells, the restrictions at each level mapped to the same region, i.e. to the left of the *I-B* subregion. Consequently, one gene (or set of genes) might control restriction at both levels. If so, T-cell recognition of major histocompatibility complex-associated antigen on macrophages and on specific B cells would be either identical or very similar.

The fact that genes mapping to the *K*-end of the *H-2* complex also control the restrictive interactions of homozygous T cells implies that F_1 T cells behave functionally as a mixture of T cells derived from the two parental strains. Positive selection to antigen in parental strain mice appears simply to alter the ratio of these two populations.

Stimulating discussion with D. B. Wilson, the technical assistance of Mrs. L. Collins, and the skillful typing of Miss K. D. Nowell are gratefully acknowledged. Thanks are extended to W. L. Elkins for the gift of recombinant mice, to N. R. Klinman for providing the anti-allotype serum and reading the manuscript, and to Mrs. D. Wilson for drawing the figure.

Received for publication 22 December 1977.

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BACILLE CALMETTE-GUÉRIN INFECTION IN THE MOUSE

Regulation of Macrophage Plasminogen Activator by T Lymphocytes and Specific Antigen*

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Experimental tuberculosis has been widely used to study cell-mediated immunity to infection (1-3) and the regulation of delayed type hypersensitivity (DTH)[1] to tuberculin and other antigens (4, 5). After infection with Bacille-Calmette-Guèrin (BCG), the host may acquire immunity to specific secondary challenge, protection against unrelated virulent organisms such as *Listeria monocytogenes* (6, 7), and also increased resistance to transplantable tumors (8). The macrophages become activated, display increased spreading and metabolic activity in culture, and may develop an enhanced capacity to kill specific as well as unrelated microorganisms. One pathway for activating macrophages depends on the generation of specifically sensitized thumus-derived (T) lymphocytes (9, 10), but little is known about the mechanism of activation or the nature and control of interactions among T cells, antigen, and macrophages.

We have reported previously that macrophages activated by various inflammatory and endocytic stimuli produce and secrete high levels of neutral protease activities including plasminogen activator (PA; 11, 12), collagenase (13), and elastase (14). Because the PA provides a sensitive measure of nonspecific macrophage activation and because antimicrobial activity of macrophages against slow growing and resistant organisms such as the tubercle bacillus is difficult to study (15), we asked whether macrophage PA could also be induced specifically, by infection with BCG.

In this report we describe the effects of systemic BCG infection in the mouse on PA activity of the peritoneal macrophages and examine the role of sensitized lymphocytes and of challenge with purified protein derivative (PPD) in regulating enzyme activity. Related studies on *Trypanosoma cruzi* infection in the mouse have been reported (16, 17).

Materials and Methods

Animals. NCS female mice, weighing 25-30 g, from The Rockefeller University were used in all studies except where noted. Inbred strains BALB/cJ and C_3H/HeJ mice were obtained from The Jackson Laboratory, Bar Harbor, Maine.

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* Supported by grants from the National Institutes of Health (AI 12975-01) and the Rockefeller Foundation.

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[1] *Abbreviations used in this paper:* BCG, Bacille Calmette-Guérin; CF, culture filtrate, *Mycobacterium tuberculosis*, strain H_{37}Rv; DTH, delayed type hypersensitivity; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; IPA, inducer of plasminogen activator; MIF, migration inhibition factor; PA, plasminogen activator; PBS, phosphate-buffered saline; PPD, purified protein derivative; T, thymus-derived.

BCG Infection. Mice were infected intravenously (10.1 ml) with 2-6 × 10^7 viable BCG (strain 1011, Trudeau Institute, Saranac Lake, N. Y.; 18). Animals were kept six mice per cage, and controls were kept under the same conditions for the same period of time. BCG was stored at −70°C and thawed once before use.

Peritoneal cells were harvested from infected or control mice at different times after infection, with or without an intraperitoneal challenge with one of the following agents 2 days before harvest: (*a*) *Mycobacterium tuberculosis,* strain $H_{37}Ra$ (lyophilized; Difco Laboratories, Detroit, Mich.). Stock suspensions were sonicated 2 mg/ml in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin and 500 μg in 1 ml PBS injected per mouse. (*b*) *M. tuberculosis,* strain $H_{37}Rv$, culture filtrate (CF; 19). This was a gift from Professor M. Chase, The Rockefeller University, and was injected (0.01-10 μg) in a solution of PBS containing 5 μg/ml Tween 80 (ICI United States, Inc., Wilmington, Del.). (*c*) Tuberculin PPD (lot no. 16, Connaught Laboratories, Toronto, Canada), 50 μg in 1 ml PBS. (*d*) Proteose peptone (Difco Laboratories), 1 ml of a 1% solution.

Cells. For the purpose of this report cells obtained from uninfected control animals without intraperitoneal challenge are termed "resident cells." Peritoneal cells were harvested by conventional procedures and were usually pooled from three to four mice. Cells were washed and suspended in culture medium consisting of Dulbecco's medium (H-21; Grand Island Biological Co., Grand Island, N. Y.) supplemented with 2% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 × 10^{-5} M mercaptoethanol (D2FBS). Cell yields from BCG-infected animals were 5-9 × 10^6 and contained 40-60% macrophages, with the remainder lymphocytes. Uninfected animals gave similar cell yields with 25-40% macrophages. Cell suspensions were distributed in multiwell Linbro plates (Linbro Chemical Co., New Haven, Conn.) with or without ^{125}I-fibrin. Unless noted otherwise each well contained 1 × 10^6 peritoneal cells of which 3-5 × 10^5 were macrophages.

Peritoneal cell cultures were either left unwashed and incubated further, with or without PPD, or were incubated for 2-4 h at 37°C and washed twice with Hanks' Balanced Salt Solution (HBSS) to remove most of the lymphocytes. These macrophage monolayers were refed D2FBS and used as described below.

Higher cell yields were obtained from infected animals challenged in vivo with the various agents listed above, 8-15 × 10^6 cells per mouse, with 5-20% polymorphonuclear luekocytes, 30-60% macrophages, and 30-50% lymphocytes. For these experiments, serial dilutions of cells were cultivated on ^{125}I-fibrin in Dulbecco's medium, antibiotics, and 20% FBS (D20FBS), with 60 μg/ml soybean trypsin inhibitor (STI fraction V, Miles Laboratories Inc., Elkhart, Ind.), to suppress fibrinolysis. The adherent cells were washed well, refed after 2-4 h, and incubated further in the same medium for at least 24 h to ensure loss of polymorphonuclear leukocytes (12).

Nylon-Wool Separation of Lymphocytes. Suspensions of peritoneal cells, 2-4 × 10^7 cells in 1-2 ml, were obtained in Dulbecco's medium + 10% heat-inactivated FBS + 5 × 10^{-5} mercaptoethanol (D10FBS) and overlayed on a nylon-wool column (300 mg); Fenwal Laboratories Inc., Morton Grove, Ill.) and preincubated with D10FBS at 37°C for 1 h (20). The column with cells was incubated at 37°C for 45 min, and the nonadherent cells eluted with 30 ml of D10FBS. The cells were washed once, counted, suspended to the desired concentration in D2FBS, and plated on radioactive Linbro plates containing a freshly plated monolayer of resident macrophages, with or without PPD. 10-25% of the peritoneal cells were recovered after passage over nylon-wool columns, of which fewer than 1% were macrophages. Cell viability, by trypan blue exclusion, was greater than 97%.

Nonadherent Cells. BCG-primed peritoneal cells were incubated in D10FBS for 1 h at 37°C, 1 × 10^7 cells per 100-mm tissue culture dish (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). The nonadherent cells were removed and pooled with cells removed by two gentle washes of the adherent monolayer. Cells were collected by centrifugation, resuspended in D2FBS, and added to monolayers of resident macrophages on ^{125}I-fibrin plates. 10-20% of peritoneal cells were recovered by this procedure with fewer than 5% macrophages.

Anti-Thy 1.2 Treatment. AKR anti-ASL_1 (anti-thy 1.2) serum was kindly provided by Dr. U. Hämmerling of the Sloan-Kettering Institute, N. Y. Peritoneal cells were obtained from four to six Balb/cJ or C_3H/HeJ mice 21-25 days after BCG infection. After washing, the cells were resuspended in RPMI-1640 + 1% FBS, 2 × 10^7/ml, divided into equal groups, and treated with antiserum or normal mouse serum (1/30 vol/vol), with or without selected rabbit serum (1/30 vol/

vol) as source of complement, and a trace amount of deoxyribonuclease. Each group of cells was incubated at 37°C for 45 min, washed, and resuspended to the same volume in D2FBS. The treated cells were then incubated in ^{125}I-fibrin plates for 1-2 days, with or without PPD, before assay. Treatment with anti-thy 1.2 serum and complement killed 24-31% of the peritoneal cells, as measured by trypan blue exclusion, and 7-16% of the cells were killed by either reagent alone.

Fibrinolysis. Fibrinolytic activity was measured either by cultivating cells directly on ^{125}I-fibrin or by a two-stage assay in which supernates, prepared by incubating cells with antigen, were added to monolayers of resident macrophages on ^{125}I-fibrin.

Direct Assay. Peritoneal cells were plated on ^{125}I-fibrin as described above and incubated at 37°C for 1-2 days with the desired concentration of PPD before assay.

Indirect Assay

PREPARATION OF SUPERNATE. Control and BCG-primed peritoneal cells were incubated in D2FBS with or without 1-50 μg PPD for 1-3 days at 37°C. Supernates were collected, centrifuged at 250 *g* for 15 min, and passed through a 0.45-μm Millex filter (Millipore Corp., Bedford, Mass.). Supernates were stable during storage at −70°C.

INDUCTION OF FIBRINOLYSIS. Resident peritoneal cells, containing 3-5 × 10^5 macrophages per well, were cultivated on ^{125}I-fibrin in D2FBS for 2 h at 37°C. The macrophage monolayers were washed twice with HBSS and incubated for 1-3 days with supernates diluted in D2FBS before assay.

^{125}I-fibrin-coated Linbro plates were prepared as described (21). Each well contained 20 μg fibrinogen and 1 × 10^5 cpm ^{125}I releasable by trypsin. Assays were run in duplicate, and appropriate controls were included in all experiments. Media were monitored for release of radioactivity before each change of medium. The assay of fibrinolysis was started by washing cells twice in HBSS followed by incubation in 0.5 ml Dulbecco's medium containing 5% dog serum which had been acid-treated to destroy inhibitors (pH 2/30 min at room temperature). 100-μl aliquots of medium were withdrawn at intervals (1-6 h) and assayed for release of radioactivity in a Packard gamma counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Under these conditions, fibrinolysis is strictly dependent on the presence of plasminogen and is therefore due to macrophage PA.

Lysozyme activity was measured as described (22).

Reagents. FBS and dog serum were obtained from Grand Island Biological Co. Endotoxin (*Salmonella abortus equi*) was a gift from Professor O. Westphal, Freiburg, Germany. Plasminogen was purified from dog serum by lysine-sepharose chromatography (23).

Results

BCG Infection and Challenge with Antigen in Vivo. The influence of systemic infection and intraperitoneal challenge with various antigenic stimuli on macrophage fibrinolysis was first examined. Peritoneal macrophages obtained from animals 3 wk after infection with 2-6 × 10^7 viable organisms spread rapidly in culture, but showed only a modest, twofold increase in fibrinolytic activity (Table I). Higher levels of macrophage fibrinolysis were observed when BCG-primed animals were challenged with specific antigen 2 days before harvest. Two soluble antigen preparations, PPD and *M. tuberculosis* CF, further enhanced activity fourfold in infected animals. A particulate antigen, $H_{37}Ra$, also resulted in a significant increase in uninfected controls, perhaps the result of phagocytic stimulation (12). Proteose peptone provided a less effective, but definite, nonspecific stimulus in infected animals. Inasmuch as nonspecific challenge with these antigens did not enhance macrophage fibrinolysis to the same extent, it seemed likely that PA was induced by an immunologically specific mechanism.

In Vitro Challenge of BCG-Primed Peritoneal Cells. We next sought conditions to provide a secondary stimulus with specific antigen in vitro.

TABLE I

*Effect of Intraperitoneal Challenge on the Fibrinolytic Activity of Cultivated Macrophages from Mice Infected with BCG**

Treatment		PA
Infection	Challenge	
		% radioactivity released/4 h/10⁶ cells
Control	Nil	2.0
	PP	1.5
	$H_{37}Ra$	6.1
	CF	1.5
	PPD	2.5
BCG	Nil	4.5
	PP	7.5
	$H_{37}Ra$	13
	CF	15
	PPD	18

* Mice were infected intravenously with 2-6 × 10^7 viable BCG. After 3 wk the animals were injected intraperitoneally with 1 ml of a 1% solution of proteose peptone (PP), 500 μg $H_{37}Ra$, 0.1 μg CF or 50 μg PPD. Peritoneal cells were harvested after 2 days and were cultivated in $D_{20}STI$ for 24 h before assay.

Peritoneal cells were obtained from mice infected 3 wk earlier with BCG, and the unfractionated population of macrophages and lymphocytes incubated on ^{125}I-fibrin with different amounts of PPD for 1 or 2 days. The cultures were then washed to remove nonadherent cells and the fibrinolytic activity of the macrophage monolayer determined. As shown in Fig. 1, the addition of PPD to BCG-primed cultures resulted in a striking dose-related enhancement of macrophage fibrinolytic activity. Stimulation of fibrinolysis was fully evident within 1 day of incubating peritoneal cells with PPD and was maximal, in this experiment, at a concentration of 2 μg/ml. A higher concentration of PPD, up to 50 μg/ml, was required for maximal fibrinolysis with cells from some groups of infected animals, and the optimal concentration of PPD was determined by titration in all subsequent experiments.

Control experiments showed that two different preparations of antigen, PPD and *M. tuberculosis* CF, could both be used for challenge in vitro and that purified endotoxin, an inducer of macrophage PA in vivo (12), did not stimulate fibrinolysis by BCG-primed cells in culture. The fibrinolysis observed after PPD stimulation was strictly dependent on plasminogen and the presence of macrophages.

From these experiments, we concluded the exposure of BCG-primed peritoneal cells to PPD in vitro provided an effective secondary stimulus for induction of macrophage PA.

Time-Course of Infection. We next examined the effect of duration of BCG infection on the enhancement of macrophage fibrinolysis by in vitro challenge with PPD. As shown in Fig. 2 b, enhancement of macrophage fibrinolysis by PPD was detectable within 2 wk, became maximal after 3 wk, and declined

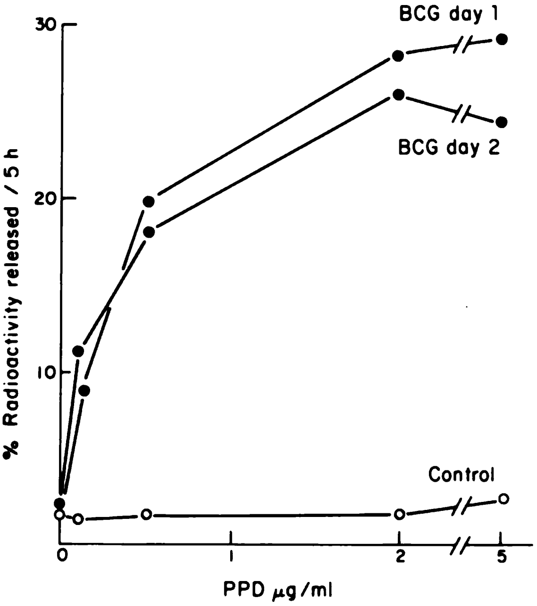

FIG. 1. Enhanced macrophage fibrinolysis after challenge of BCG-primed cells with PPD in vitro. Peritoneal cells were harvested 26 days after infection with BCG or from control, uninfected animals. Cells were cultivated for 1 or 2 days on ^{125}I-labeled fibrin plates in D2FBS with or without PPD, washed, and the resulting macrophage monolayer assayed for fibrinolytic activity.

subsequently. Enlargement of spleen (Fig. 2 a) and liver followed a roughly similar time-course, but was noted earlier and diminished more slowly with time. Fig. 2 c shows further that the three to fourfold stimulation of fibrinolysis by PPD was selective, whereas lysozyme secretion by the same macrophages was unaffected.

Sensitized Lymphocytes and PPD Induce Fibrinolysis in Resident Macrophages. To learn more about the nature of the antigen-sensitive cells, we next separated the lymphoid and macrophage elements of the BCG-primed peritoneal cavity. Cells were passed over nylon-wool columns to remove macrophages, and nonadherent lymphocytes were then co-cultivated with control resident macrophages with or without PPD. After incubation for 1 or 2 days, the lymphocytes were removed by washing, and macrophage fibrinolysis assayed. Figs. 3 and 4 illustrate experiments in which nylon wool purified sensitized lymphocytes and PPD stimulated fibrinolysis by resident macrophages approximately fourfold, whereas lymphocytes from uninfected animals had no such activity. In control experiments, not shown, BCG-primed lymphocytes displayed no fibrinolytic activity in the absence of macrophages and died within 1-2 days in culture.

Induction of fibrinolysis in resident macrophages was proportional to the dose of PPD (Fig. 3) and to the number of sensitized lymphocytes (Fig. 4). The optimal concentration of PPD varied from 5 to 50 μg/ml, and enhanced fibrinolysis could be detected at a lymphocyte: macrophage ratio of 1:40, although maximal stimulation was observed at a ratio of 1:1. Co-cultivation of

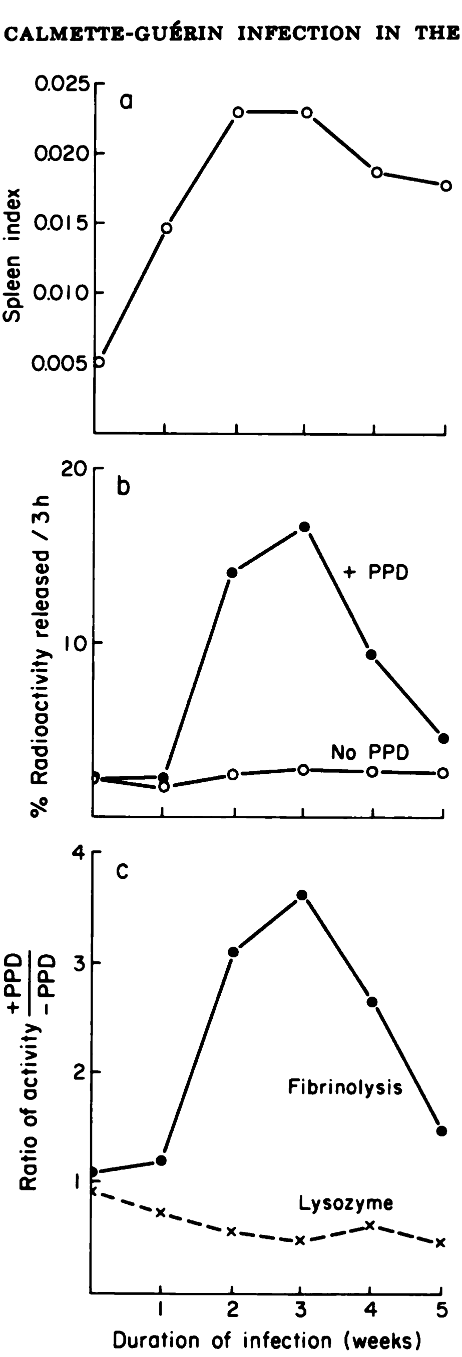

FIG. 2. Effect of duration of BCG infection on enhancement of macrophage fibrinolysis by PPD. Groups of mice were infected at weekly intervals with BCG, 2-6 × 10^7 viable organisms, intravenously. Peritoneal cells were cultivated on ^{125}I-fibrin plates, 1 × 10^6 cells/well, in the presence or absence of 10 μg/ml PPD, for 1 day. Cultures were then washed, and macrophage fibrinolysis and lysozyme secretion determined. (a) Spleen index; (b) fibrinolysis; (c) ratio of fibrinolysis and lysozyme secretion with and without PPD.

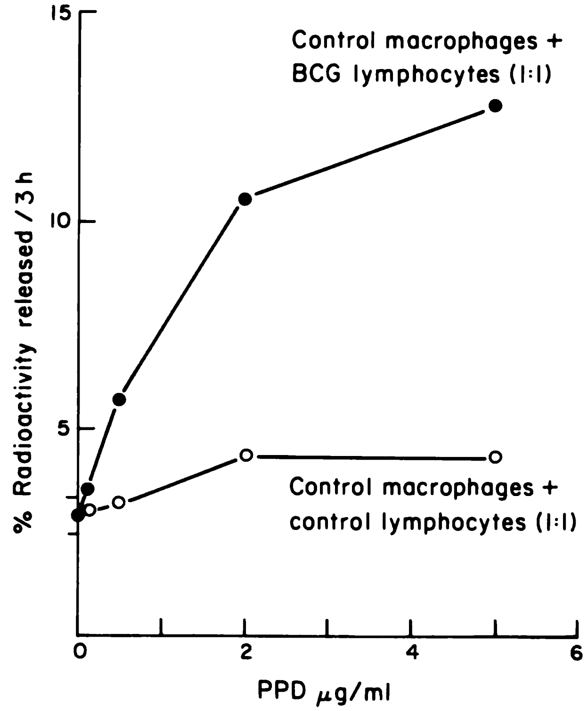

FIG. 3. Stimulation of fibrinolysis in resident macrophages by BCG-primed lymphocytes and PPD. Nylon-wool-separated lymphocytes were prepared 26 days after infection or from control, uninfected animals. 4 × 10^5 lymphocytes were added to ^{125}I-fibrin-coated plates containing 4 × 10^5 resident macrophages/well and incubated in D2FBS with different concentrations of PPD, for 2 days, before assay.

sensitized lymphocytes and resident macrophages with PPD for 1 or 2 days gave similar results.

The enrichment of antigen-sensitive lymphocytes in BCG-primed cell populations is shown in Table II and compares the induction of macrophage fibrinolysis by unfractionated peritoneal cells, nylon-wool separated lymphocytes, and lymphocytes separated by nonadherence to a tissue culture dish. Cell recovery for nylon-wool separated and nonadherent lymphocytes was 11 and 6%, but recovery of PPD-dependent induction of fibrinolysis was 33 and 27% for each fraction, indicating a possible enrichment of three- to fourfold in specific activity. This estimate is approximate because of the intrinsic activity of macrophages in the various fractions.

Role of Thymus-Derived (T) Lymphocytes. Induction of macrophage fibrinolysis by infection with *T. cruzi* (17) requires T lymphocytes. To establish whether T cells also play a part in BCG infection, peritoneal cells obtained 3 wk after infection were exposed to anti-thy 1.2 antiserum with or without complement and then incubated in the presence or absence of PPD for 1 day before assay. As shown in Table III, pretreatment with anti-thy 1.2 antiserum with complement prevented the PPD-dependent stimulation of macrophage fibrinolysis, whereas treatment with either reagent alone resulted in three- to fivefold enhancement of activity. The low levels of PPD-independent activity were

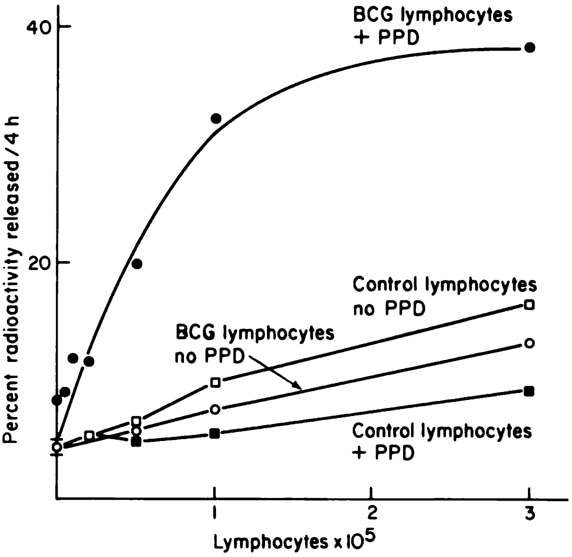

FIG. 4. Dose response of stimulation of macrophage fibrinolysis by BCG-primed lymphocytes with PPD. Nylon-wool-separated lymphocytes were obtained from control, uninfected animals or 24 days after BCG infection. Suspensions of lymphocytes were added to monolayers containing 3 × 10^5 resident macrophages/well and cultivated in D2FBS, with or without 20 μg PPD, for 2 days, before assay. (●—●) BCG lymphocytes with PPD: (○—○) BCG lymphocytes without PPD; (■—■) control lymphocytes with PPD; (□—□) control lymphocytes without PPD.

not influenced. Induction of macrophage fibrinolysis by PPD in BCG infection therefore depends on sensitized T lymphocytes.

Supernatant Inducer of Macrophage PA. Lymphoid cultures release soluble mediators which enhance macrophage fibrinolysis after sensitization and specific challenge (17) or after lectin stimulation (24, 25). Peritoneal cells from BCG-infected and control animals were therefore incubated with PPD, and the supernate added to resident macrophages. Fig. 5 a illustrates an experiment in which supernate from BCG-primed cells challenged with PPD enhanced macrophage fibrinolysis more than fourfold, whereas control supernate or medium showed no such effect. A dose-response experiment (Fig. 5 b) showed that stimulation of fibrinolysis was maximal at a supernatant concentration of 25-50%, but was detectable at 2.5% vol/vol.

Because the fibrinolysis was dependent on plasminogen, we postulate that the active supernate contained an inducer of PA (IPA). Production of IPA was detectable within 1 day of antigen challenge, but increased with time and was maximal after 2-3 days. Optimal levels of IPA were produced at low concentrations of serum, 1-5% FBS or 1-10% horse serum. Higher concentrations of FBS were strongly inhibitory. Heat inactivation of serum (56°C/30 min) had no effect nor did the addition of mercaptoethanol. Although sensitized cells yielded the highest levels of IPA activity after challenge with PPD, some preparations contained low but significant levels of activity without PPD. Spleen cultures from BCG-infected animals also generated IPA after stimulation with PPD.

The effects of supernate containing IPA on resident macrophages were also

TABLE II

*Stimulation of Fibrinolysis of Resident Macrophages by BCG-Primed Peritoneal Cells and PPD**

Treatment	Cell yield 10^5/mouse	Macrophage fibrinolysis			
		−PPD		+PPD	
		sp act U/10^5 cells	*yield* U/mouse	*sp act* U/10^5 cells	*yield* U/mouse
Peritoneal cells	61	0	0	1.9‡	12
Nonadherent	3.6	0.60	0.22	9.0	3.2
Nylon wool	6.6	0.50	0.33	6.0	4.0

* Peritoneal cells were harvested from mice 22 days after infection with BCG. Serial dilutions of total peritoneal cells, nonadherent and nylon-wool-separated lymphocytes were added to monolayers of resident macrophages with or without PPD (10 μg/ml) and cultivated for 2 days before assay.

‡ Fibrinolytic activity of peritoneal cells without resident macrophages subtracted (1.7 U/ 1 × 10^5 cells).

TABLE III

*Effect of Anti-Thy 1.2 Antiserum and Complement on Stimulation of Macrophage Fibrinolysis by BCG Infection and PPD**

Treatment	Macrophage fibrinolysis	
	−PPD	+PPD
	% radioactivity released/4 h	
Anti-thy 1.2	3.4	19
Normal mouse serum + rabbit complement	4.9	15
Anti-thy 1.2 + rabbit complement	4.5	5.7

* Peritoneal cells were obtained from six male Balb/c mice 21 days after infection with BCG. After treatment with antiserum and complement (see Materials and Methods), 4 × 10^5 cells were incubated on ^{125}I-fibrin with or without 50 μg PPD for 1 day before assay.

studied. Enhanced fibrinolysis was noted after 1 day, but increased with duration of exposure and was maximal at 3 days. Macrophages that had been exposed to active supernates spread more extensively than controls, showed active membrane ruffling, and accumulated numerous phase-dense granules in the perinuclear region. Such activated resident macrophages did not show the toxicity sometimes seen with macrophages from BCG-infected animals after PPD challenge in the presence of sensitized lymphocytes. Lysozyme secretion by macrophages was not enhanced by exposure to supernates rich in IPA (not shown).

Discussion

BCG infection in the mouse elicits a variety of humoral and cellular responses, among which effects on lymphocyte and macrophage function feature prominently (1–5). The present studies show that high levels of macrophage

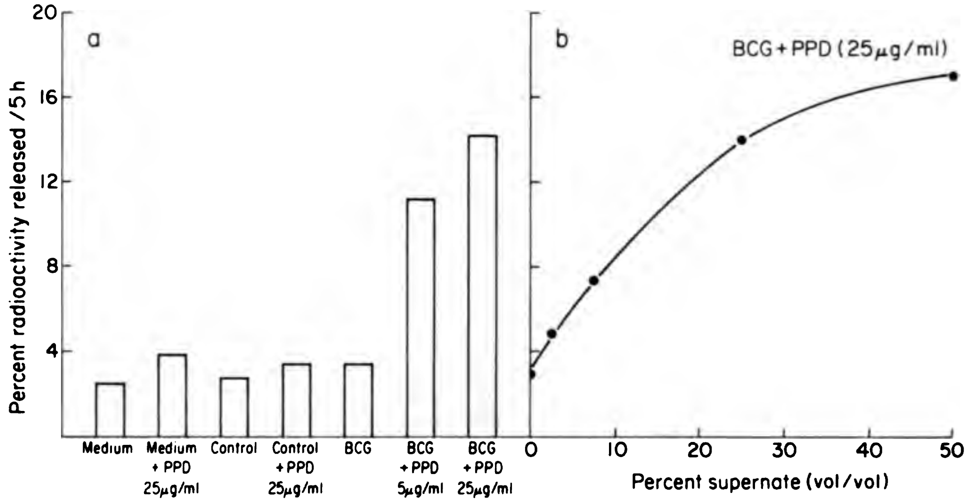

FIG. 5. Induction of macrophage fibrinolysis by peritoneal cell supernates after BCG infection and PPD. Cells obtained from control animals or 30 days after infection with BCG were cultivated for 2 days on nonradioactive Linbro plates 1 × 10^6 cells/well, in D2FBS, with or without PPD. Supernates were then added at different concentrations to resident macrophages on ^{125}I-fibrin plates, 4 × 10^5/well, for 3 days before assay. (a) Macrophage fibrinolysis, concentration of supernates 25% vol/vol; (b) dose response of supernate prepared from BCG-primed cells after challenge with 25 μg/ml PPD.

fibrinolysis are induced under conditions that parallel the expression of cell-mediated immunity and DTH (26). Enhanced fibrinolysis can be elicited by specific challenge of BCG-primed animals in vivo or of sensitized peritoneal cells directly in culture. The antigen-sensitive response depends on T-lymphocytes that may stimulate fibrinolysis by macrophages from uninfected and infected animals either after co-cultivation or by the release of soluble mediators. Enhanced fibrinolysis is due to macrophage PA and is regulated by an immunologically specific mechanism, as shown by these and related studies with *T. cruzi*. Although highest levels of macrophage fibrinolysis were observed when BCG- and *T. cruzi*-immune animals were challenged with specific antigen, nonspecific stimulation of PA may follow direct challenge of primed macrophages with a particulate stimulus. The present system offers the advantage of a soluble antigen. Further evidence for immunologically specific regulation of macrophage PA has been provided by studies of mixed leukocyte culture reactions in the mouse[2] and of hypersensitivity to PPD in guinea pigs immunized with Freund's adjuvant (25).

Our results draw attention to the role of antigen restimulation and of T cells in control of macrophage activation. No significant fibrinolysis was found without secondary challenge, although the peritoneal cavity of BCG-infected mice contained substantial numbers of sensitized T cells, as noted previously by others (27). These sensitized lymphocytes responded rapidly to PPD, and measurement of macrophage fibrinolysis provided a sensitive and quantitative measure of their generation in the peritoneal cavity during infection. In the course of infection, the sensitization of peritoneal lymphocytes becomes apparent somewhat later and declines more rapidly than enlargement of the spleen and liver. It is possible that sensitized cells persist longer in the spleens of BCG-infected animals than after infection with *T. cruzi* (17). The decline of PPD-stimulated fibrinolysis by peritoneal cells at later stages of infection could be due to either loss or inhibition of sensitized T cells.

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[2] Newman, W., S. Gordon, U. Hämmerling, and B. R. Bloom. Manuscript submitted for publication.

Induction of macrophage PA by specific antigen depends on T lymphocytes in BCG infection, as with *T. cruzi* (17) and the mixed leukocyte culture reaction (see fn. 2). T cells are known to play a role in antimicrobial protection in BCG-infected mice (3, 27) and rats (28) and in development of DTH to tuberculin (29, 30). Nothing is known, however, about the frequency, heterogeneity, or proliferative characteristives of T cells that regulate macrophage fibrinolysis or their relation to protective and memory cells generated by BCG.

Stimulation of fibrinolysis by BCG infection provides a particularly useful model system to study interactions between sensitized lymphocytes, antigen, and macrophages under defined conditions in culture. Titration experiments with nylon-wool-separated lymphocytes and active supernates showed that direct contact and soluble mediators stimulated macrophage fibrinolysis to a similar extent. The 2-stage assay offers the possible advantage of separating production of a PA inducer from its action on macrophages, although it is likely that macrophages also play a role in secondary stimulation of sensitized T cells by antigen (31, 32). A particular advantage of the BCG model, however, is that sensitized lymphocytes and PPD can be titrated directly on macrophages from uninfected animals. The sensitivity of the fibrinolytic assay is greatly enhanced by serum-free conditions, and it may be possible to determine the activity and number of sensitized peritoneal lymphocytes by limiting dilution analysis.

The nature of the inducers of macrophage PA remains obscure, although it is known that lymphocytes release similar products after stimulation with lectins (24, 25). The IPA generated by MLC reactions closely resembles migration inhibition factor in activity, size, and in production by both Ly-1 and Ly-2,3 subsets of T lymphocytes (see fn. 2). Other workers have reported that PA produced by transformed fibroblasts is itself able to generate MIF-like activity from undefined serum components (33). The supernatant inducer of macrophage PA studied in the present report cannot be PA itself because no fibrinolytic activity could be detected before incubation with macrophages and because medium conditioned by thioglycolate broth-stimulated macrophages, a rich source of PA, did not enhance fibrinolysis by unstimulated macrophages. The relationship between IPA, MIF, PA, and serum zymogens clearly deserves further study.

Products of stimulated lymphocytes have several effects on macrophages other than stimulating PA and inhibiting migration (34). Responses of macrophages reported include enhanced secretion of collagenase (35), C_2 (36), and pyrogens (37), increased plasma membrane spreading, ingestion of EIgMC via receptors for C_3b,[3] and formation of giant cells (38). The present studies show that lysozyme production is, however, not influenced. Purification of IPA is necessary to determine whether the multiple macrophage responses are mediated by a common molecular mediator.

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[3] Bianco, C., S. Gordon, and B. Bloom. Unpublished observations.

Induction of macrophage PA by T cells provides an important mechanism to amplify lymphocyte function, to generate various mediators of inflammation, and may also contribute to connective tissue catabolism and tissue injury associated with DTH in tuberculosis. Activation of macrophages by this pathway can be augmented by endocytic stimuli and by humoral mediators generated by activation of complement and coagulation cascades (39). The mechanism by which BCG-activated macrophages could acquire antimicrobial and antitumor cell activity, however, remains unknown.

Summary

High levels of plasminogen activator (PA) were induced in mouse peritoneal macrophages by infection with BCG, 2–6 × 10^7 viable organisms intravenously, followed 3–4 wk later by intraperitoneal challenge with purified protein derivative (PPD) 2 days before harvest. Macrophages obtained from infected animals without boosting showed little fibrinolytic activity, but challenge of Bacille-Calmette-Guèrin (BCG)-primed peritoneal cells with PPD in culture also enhanced macrophage PA 4- to 10-fold. Stimulation of macrophage PA by PPD depended on specifically sensitized thymus-derived (T) lymphocytes because it was abolished by pretreatment of BCG-primed peritoneal cells with anti-thy 1.2 antiserum and complement. A direct assay was developed in which nylon wool separated sensitized lymphocytes and PPD induced PA in macrophages from uninfected animals under defined conditions on ^{125}I-fibrin. Enhanced macrophage fibrinolysis was proportional to concentration of PPD and the number of sensitized lymphocytes transferred. An indirect two-stage assay was also used to show that BCG-sensitized peritoneal cells released a soluble inducer of macrophage PA into the culture medium, after challenge with PPD.

Induction of macrophage PA by PPD challenge in vitro made it possible to study the generation and activity of sensitized peritoneal lymphocytes at different stages of infection. Our results show that nonadherent peritoneal cells of BCG-infected mice provide a rich source of specifically sensitized lymphocytes and that macrophage activation is limited by continued availability of antigen, as well as sensitized lymphocytes. Induction of macrophage PA provides a sensitive, versatile, and rapid in vitro assay to study the role of lymphocytes and specific antigen in macrophage activation by BCG.

We thank Eileen Fontanetta, Renee Zweibel, and Ann Chin for their assistance.

Received for publication 14 December 1977.

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HEMATOPOIETIC STEM CELL REGULATORY VOLUMES AS REVEALED IN STUDIES OF THE bg^J/bg^J:W/W^v CHIMERA*

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Foremost among the open questions of hematopoiesis is the nature of the mechanisms that govern the behavior of pluripotent stem cells. Several types of evidence suggest that stem cell activities are directed by local control systems. Local regulation is apparently expressed in the centripetal movement of active marrow during postnatal growth of many species, in the predilection of hematopoietic foci for endosteal surfaces (1), in the selective distribution of erythroid and granuloid colonies arising from stem cells implanted in X-irradiated mice (2), and in the microenvironmental defect seen in genetically anemic mice carrying the mutant steel locus (3, 4). The effects of adherent cells derived from marrow as seen in heterotopic implants (5) and in bone marrow cultures (6) are also strongly suggestive of a regulatory role of bone marrow-associated elements in the activities of pluripotent stem cells. Because the W/W^v mouse has very few macrocolony-forming stem cells (CFU_S)[1] but an apparently normal marrow microenvironment, this genotype may be useful for studying the interaction between CFU_S and their immediate environment. This follows from the comparative ease with which CFU_S from +/+ littermates or congenic mouse strains can be transplanted to W/W^v mice without irradiation or other treatment (7–9). We are concerned here with the evolution of bone marrow chimerism in the W/W^v mouse as a function of marrow transplantation dose. The results of this study point to the existence of discrete stem cell regulatory vol of about 10^8 μm^3, a dimension consistent with concepts of short-range cell-cell interactions.

Materials and Methods

We chose histocompatible bg^J/bg^J (beige) mouse marrow as the source of CFU_S for the W/W^v mouse because the giant sudanophilic granules (>1 μm) characteristic of bg^J/bg^J neutrophils provide a convenient marker of W/W^v marrow replacement (8). W/W^v and bg^J/bg^J mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. Both male and female bg^J/bg^J mice, 2–4 mo of age and weighing 20–25 g, were used as marrow donors, but only male W/W^v mice of similar age weighing 15–20 g were used as bone marrow recipients because of incompatibility of a male graft in a female host. Donor marrow was obtained from a femur shaft, suspended in Hanks' balanced salt solution for total nucleated cell counts, and then serially diluted with additional Hanks' solution to the desired cell concentrations. Each W/W^v recipient was injected with 0.5 ml of the appropriate bg^J/bg^J marrow cell suspension by tail vein.

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* Supported by the U. S. Department of Energy.

[1] *Abbreviation used in this paper:* CFU_S, macrocolony-forming stem cells.

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Peripheral blood samples were obtained from each recipient W/W^v mouse at various times up to 720 days. For detection of beige neutrophils, blood smears were fixed in formaldehyde vapor, stained with Sudan black B, and counterstained with Gurr's Giemsa (10). A minimum of 100 neutrophils on each blood smear was scored for the presence of giant sudanophilic granules. To determine whether temporal changes in the concentration of bg^J/bg^J neutrophils mirrored the takeover of W/W^v marrow by the implanted CFU$_S$, we also performed spleen colony assays in selected mice. For this purpose, 0.5 ml of femoral marrow cell suspensions prepared from bg^J/bg^J:W/W^v chimeras was given intravenously to each of 5–10 CBA/J mice 2 h after their X-irradiation with 1,000 rads. The recipients were killed 7 days later and surface colonies were counted with the aid of a dissecting microscope after fixation of the excised spleen. The number of discrete surface colonies was corrected for endogenous colonies and the basal CFU$_S$ concentration in W/W^v mice (0.4 ± 0.06 per 10^5 nucleated cells).

The fraction of injected bg^J/bg^J CFU$_S$ present in a W/W^v femur at 24 h (seeding efficiency) was determined by secondary transplantation to irradiated recipients and spleen colony assay as described above. Seeding efficiency is thought to be independent of the injected dose up to at least 90×10^6 nucleated marrow cells (11). The initial bg^J/bg^J CFU$_S$ uptake was related to W/W^v marrow volume, determined by agar replacement (12). Femoral medullary cavity volume was estimated by comparison of the weight of a femoral shaft after removal of its marrow content and replacement with 2% agar. It is known that a femur represents 6% of the total marrow in a mouse (13).

The possible contribution of bg^J/bg^J extramedullary hematopoiesis was examined by determination of neutrophil replacement in W/W^v mice splenectomized 24 h after injection of bg^J/bg^J marrow cells. Redistribution of intravenously injected CFU$_S$ among the various tissues of an X-irradiated mouse is known to be completed by 16–24 h (14).

Results

The concentration of CFU$_S$ in bone marrow is at least 50–100 times greater in the bg^J/bg^J than in the W/W^v mouse. We determined that bg^J/bg^J marrow contains 24 ± 3 CFU$_S$ per 10^5 nucleated cells and that 1.3 ± 0.4% of the transplanted CFU$_S$ could be recovered from the marrow of a W/W^v femur 24 h later. When the CFU$_S$ concentration is corrected for a seeding efficiency of 10% in the spleen colony assay (15–17), it follows that injection of 10^5 bg^J/bg^J bone marrow cells led to the delivery of about three CFU$_S$ to the marrow in each femur. Because a femur contains 6% of the marrow of a mouse, about 50 CFU$_S$ were therefore delivered to the total marrow. By using the agar replacement technique, we found that the femoral medullary volume in a 20-g W/W^v mouse was 16×10^9 μm^3, which can be extrapolated to a total marrow vol of about 300×10^9 μm^3 or 0.3 ml.

The replacement of W/W^v blood neutrophils by bg^J/bg^J neutrophils after transplantation of bg^J/bg^J marrow cells reflects the growth of CFU$_S$ in the chimeric mice (Fig. 1). The degree of replacement was linearly related to the bone marrow CFU$_S$ concentration over the range of 5–95% bg^J/bg^J neutrophils without a change in overall neutrophil concentration. Therefore, the peripheral blood percentage of bg^J/bg^J neutrophils provides a convenient parameter for assessment of temporal changes in the growth and development of implanted stem cells in a given animal. With marrow cell doses of 0.2×10^5–1×10^5, bg^J/bg^J blood neutrophils were found in only 3 of 20 recipient W/W^v mice even after 300 days: 2 of 5 with 0.2×10^5, 0 of 5 with 0.4×10^5, 1 of 5 with 0.5×10^5, 0 of 5 with 1×10^5. However, with doses of 2×10^5 or more, bg^J/bg^J neutrophils were seen in all of 65 recipient mice.

Beige neutrophils appeared in the peripheral blood after a latency period of

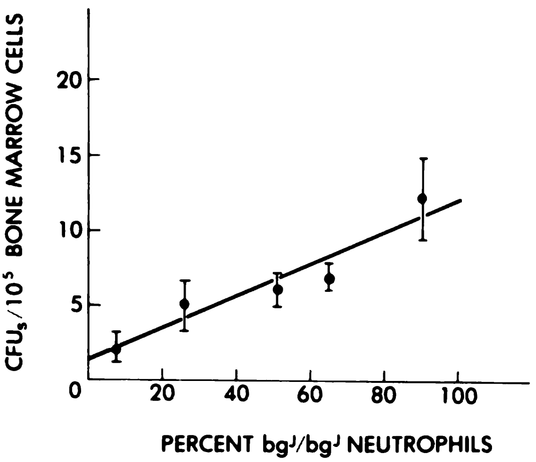

FIG. 1. Relationship between bone marrow CFU$_S$ concentration and bg^J/bg^J blood neutrophil percentage in W/W^v recipients of bg^J/bg^J bone marrow. (Symbols designate mean ± SE for four to seven mice; CFU$_S$ concentration uncorrected for seeding efficiency in spleen colony assay. Correlation coefficient, 0.91; $P < 0.025$.)

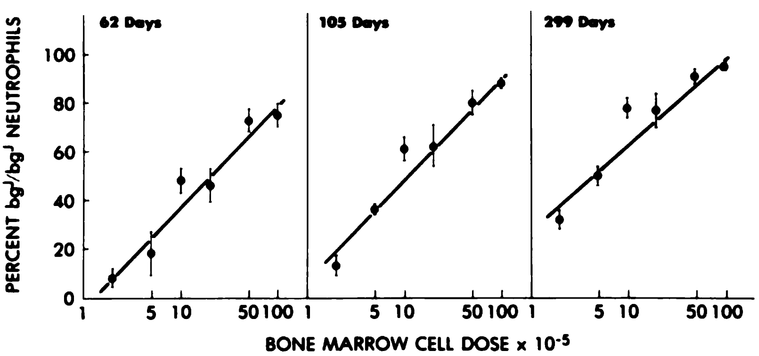

FIG. 2. Replacement of W/W^v blood neutrophils by bg^J/bg^J neutrophils as a function of marrow inoculum dose and time. (Symbols designate mean ± SE for four to eight mice; the same mice were sampled at each time interval. Correlation coefficient at 62 days, 0.97; at 105 days, 0.97; at 299 days, 0.95; $P < 0.005$.)

2–3 wk, the rate of appearance depending on the dose of bg^J/bg^J marrow cells injected. In one experiment, the replacement of W/W^v neutrophils by beige neutrophils was followed in each recipient mouse by sampling at mean times of 62, 105, and 299 days after intravenous bone marrow cell doses of 2 × 10^5–100 × 10^5. As shown in Fig. 2, the results are consistent with a linear log dose-response, with 50% replacement doses (D_{50}) of 20 × 10^5, 10 × 10^5, and 4 × 10^5 bg^J/bg^J bone marrow cells at 62, 105, and 299 days, respectively. In a second experiment, W/W^v neutrophil replacement was determined by sampling a few mice repeatedly during a period of 720 days after transplantation of either 2 × 10^5 or 2 × 10^6 bg^J/bg^J bone marrow cells. At the lower dose, beige neutrophil concentration increased linearly at a rate of 0.1% per day, the time for 50% replacement (T_{50}) being 450 days (Fig. 3). At the higher dose, beige neutrophils initially increased rapidly and then progressively more slowly, the overall

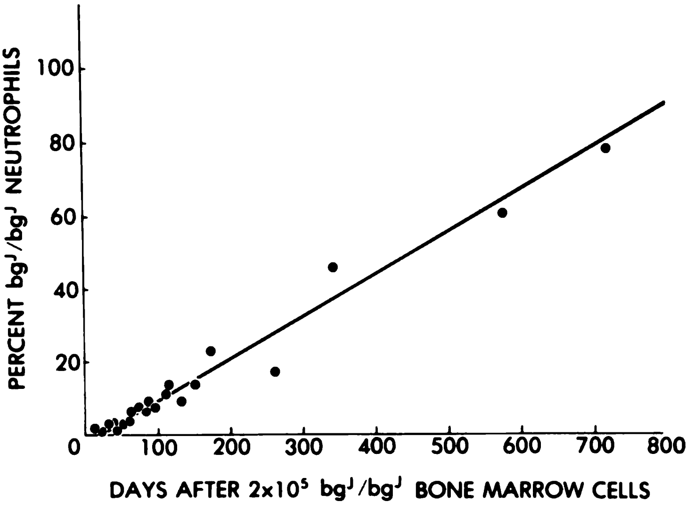

FIG. 3. Blood neutrophil replacement with time after intravenous injection of 2×10^5 bg^J/bg^J bone marrow cells. (Each symbol designates the mean value in two mice sampled repeatedly. Correlation coefficient, 0.98; $P < 0.001$.)

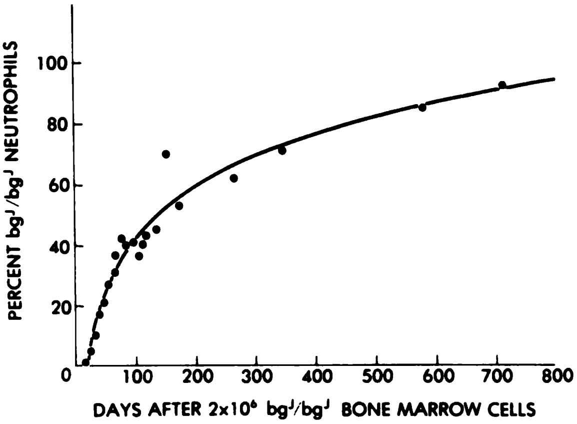

FIG. 4. Blood neutrophil replacement with time after intravenous injection of 2×10^6 bg^J/bg^J bone marrow cells. (Each symbol designates the mean value in two mice sampled repeatedly. Correlation coefficient, 0.99; $P < 0.001$.)

hyperbolic relationship being consistent with a linear log time-response (Fig. 4); the T_{50} was 140 days, a reduction by only a factor of three despite a 10-fold increase in the transplantation dose.

The replacement of W/W^v neutrophils was not altered significantly by splenectomy 24 h after injection of 50×10^5 bg^J/bg^J bone marrow cells. At 10 wk, the mean bg^J/bg^J blood neutrophil percentage was 86 ± 2 in six splenectomized W/W^v recipients compared to 91 ± 2 in five control mice. Because the spleen is known to be an important repository of intravenously injected CFU_S, this finding suggests that essentially all of the relevant stem cells were delivered to the marrow within a day after bg^J/bg^J bone marrow cell injection.

Discussion

The W/W^v mouse presents a macrocytic anemia, apparently related to a deficiency of CFU_S along with a deficiency of a "theta-sensitive" regulatory cell (9, 19). Nevertheless, this genotype has a fairly cellular bone marrow with normal concentrations of early erythroid (CFU_E) and granuloid (CFU_C) progenitors (20, 21), a reasonably normal neutrophil reserve (22), and normal blood neutrophil concentration (22–24). Despite the anemia, overall rates of replacement of W/W^v erythrocytes and neutrophils by bg^J/bg^J erythrocytes and neutrophils are generally similar after bone marrow transplantation (8). The correlation described here between the increase of donor marrow stem cells (CFU_S) and the replacement of W/W^v neutrophils by neutrophils containing the bg^J/bg^J cytoplasmic marker provides further evidence of the appropriateness of the marker as an indicator of the takeover of W/W^v marrow by the implanted bg^J/bg^J stem cells.

The present studies, covering a 50-fold range of bg^J/bg^J inoculum doses and a 2-yr period of observation, reveal a hyperbolic pattern of W/W^v blood neutrophil replacement that conforms to a linear log dose-response. To interpret the hyperbolic relationships observed in the takeover of W/W^v marrow by bg^J/bg^J CFU_S, it is necessary to consider the competency of the injected CFU_S and their initial distribution and secondary colonization within the marrow volume. CFU_S assays are reported to be similar in X-irradiated and unirradiated W/W^v mice (17, 18); hence, competency of the bg^J/bg^J CFU_S would seem to be independent of the presence of disadvantaged W/W^v stem cells. Although our low-dose studies thus far might signify that all of the marrow-implanted CFU_S were not competent with respect to W/W^v blood neutrophil replacement, further work is necessary to determine the extent to which this may reflect neutrophil counting statistics and the observation period. Extrapolation of the log dose-response slopes to the X (dose) axis (Fig. 2) points to a limiting bone marrow cell dose of 1.1×10^5 (55 marrow-implanted CFU_S) at 62 days, 0.7×10^5 (35 marrow-implanted CFU_S) at 105 days, and 0.14×10^5 (7 marrow-implanted CFU_S) at 299 days. The probability of activation of one or another implanted CFU_S no doubt depends on various factors, e.g., its location in relation to bone surfaces (25) and the number deposited within a specified volume. However, despite any heterogeneity of discrete regions within the marrow as a whole, the supposition that a single bg^J/bg^J CFU_S can eventually overcome proximate W/W^v stem cells would appear to provide a reasonable starting point for analysis of the bone marrow cell dose-response relationships in the development of bg^J/bg^J:W/W^v chimerism.

Because medullary sites in a mouse are completely hematopoietic, the injected CFU_S should be distributed within the total marrow volume. In contrast to the report by Lord et al. (25) of a normally occurring CFU_S gradient from the bone surface to marrow axis, we have found a quite uniform distribution throughout the femoral marrow (26). Although there may be some selectivity of the initial marrow distribution when CFU_S are introduced rather abruptly into the peripheral circulation, as a first approximation it seems reasonable to assume an overall random distribution. Thus, the initial seeding of marrow by bg^J/bg^J CFU_S might be expected to conform to statistics of random

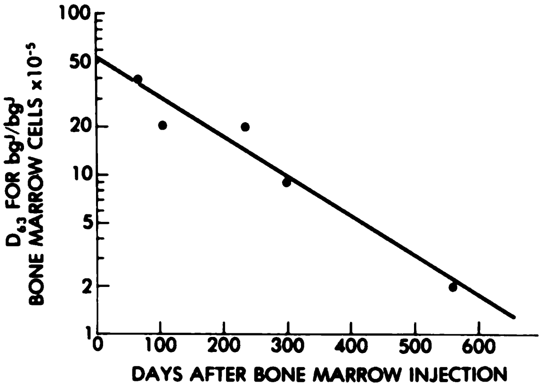

FIG. 5. The bg^J/bg^J bone marrow cell dose for 63% replacement of W/W^v blood neutrophils. (Correlation coefficient, 0.97; $P < 0.005$.)

sampling in which the probability of no seeding in a sampling unit or target volume will be given by $e^{-\lambda}$ where λ represents the mean occurrence of CFU_S per target volume after a particular bone marrow cell dose. The probability of seeding in a target volume $(1 - e^{-\lambda})$ will equal 0.63 when λ corresponds to a mean of one CFU_S per volume. Accordingly, the bg^J/bg^J bone marrow cell dose required for 63% replacement of W/W^v neutrophils (D_{63}) should correspond to the seeding of an average of one bg^J/bg^J CFU_S per target volume after correction for subsequent (secondary) colonization.

The temporal decrease in D_{63} derived from the data presented in Figs. 2–4 provides a basis for distinguishing the immediate seeding of CFU_S from the subsequent migration. The D_{63} decreased exponentially with a half-time of 120 days as a reflection of secondary colonization by the progeny of initially deposited CFU_S (Fig. 5). Hence, the Y axis intercept of 52×10^5 bg^J/bg^J bone marrow cells should approximate the theoretical D_{63} before bg^J/bg^J CFU_S migration to neighboring marrow microenvironments. This D_{63} corresponds to an uptake by bone marrow of about 2,600 CFU_S. If single-hit kinetics prevail, the sampling unit or target volume when $\lambda = 1$ will be 1.1×10^8 μm^3 (bone marrow volume, or 300×10^9 μm^3 divided by D_{63}-equivalent CFU_S, or 2,600). A Poisson plot derived from a target vol of 1.1×10^8 μm^3 is shown in Fig. 6 in relation to intercepts of least squares regressions computed for bg^J/bg^J isoeffect doses (D_{20}, D_{30}, etc.) as a function of time. The various isoeffect dose-time intercepts are in reasonable agreement with the Poisson prediction, which does not, of course, take into account biological variability. Target volumes may differ, for example, in the number of hits required for successful repopulation and thus a model based on a single hit with variable target size may provide a more precise representation.

Our analysis leads to the hypothesis that mouse bone marrow is compartmentalized into essentially self-contained stem cell regulatory volumes or domains equivalent on the average to about 50 cell diameters. This dimension is consistent with the presumptive role of short-range cell-cell interactions in the regulation of pluripotent stem cells as seen, for example, in studies with the Sl/Sl^d mouse (4). Among the factors determining the dimensions of the stem cell domains may be the distribution of putative regulatory cells of the marrow

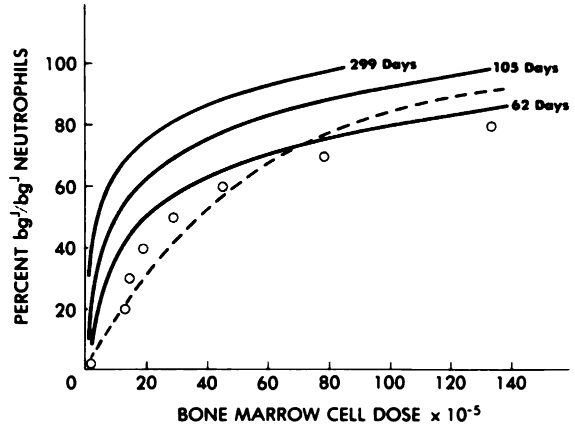

FIG. 6. Bone marrow cell dose and time parameters for blood neutrophil replacement in the bg^J/bg^J:W/W^v chimera in relation to Poisson expectation. (Solid lines are derived from data in Fig. 2; dashed line depicts Poisson plot with symbols designating intercepts of iso-effect dose-time linear regressions.)

stroma and the nature of the marrow vasculature, about which much more needs to be learned. Although each stem cell regulatory volume is undoubtedly subject to extrinsic influences, the concept of a discrete functional unit in which stem cell proliferation is geared to the density of the stem cell population provides a framework for understanding control of the stem cell population in marrow as a whole. On a broader scale, we have shown that the cellularity of distinct marrow areas is also locally controlled (27).

Our analysis of blood neutrophil replacement patterns in the bg^J/bg^J:W/W^v chimera also suggests that the local traffic of stem cells is ordinarily quite limited and restricted to proximate target or regulatory volumes, as in relays. If colonization were a fairly rapid event, only a week or so would have been required for the six doublings necessary to overcome the 50-fold difference in the number of transplanted bg^J/bg^J CFU_S. Yet secondary colonization by migrant CFU_S was clearly a slowly evolving process in the W/W^v marrow; only about 80% of the W/W^v blood neutrophils were replaced 2 yr after transplantation of 2 × 10^5 bg^J/bg^J bone marrow cells, whereas a similar degree of replacement occurred 2 mo after transplantation of 100 × 10^5 bg^J/bg^J bone marrow cells. Significantly, this limitation of CFU_S migration is not seen in a severely hypocellular marrow. Marrow regeneration in a radiation chimera is also a hyperbolic function of the marrow transplantation dose (28), but the rate of regeneration is much faster than the rate of replacement of W/W^v marrow by implanted bg^J/bg^J stem cells. Apparently, the more rapid expansion of the donor stem cell population and the decreased constraint to stem cell movement in a radiation-induced hypocellular marrow facilitates secondary colonization from the initially seeded sites. In a preliminary study, we have observed a similar result in bg^J/bg^J:W/W^v chimeras after treatment with hydroxyurea. The discontinuous nature of local stem cell migration would appear to be an important consideration in the marrow transplantation dose required for various clinical applications.

Summary

The kinetics of bone marrow replacement was studied in W/W^v mice implanted with bg^J/bg^J (beige) stem cells, with the characteristic beige neutrophil marker as a criterion of the takeover of host marrow by donor marrow. A hyperbolic pattern of W/W^v marrow replacement conforming to a log dose-response was observed in experiments encompassing a 50-fold range of bg^J/bg^J inoculum doses and a 2-yr period of observation. The dose-response relationships were consistent with random seeding of stem cells in the host marrow coupled with a decreasing efficiency of secondary colonization by local migration. Application of single-hit Poisson sampling statistics to the dose-response data led to the hypothesis that mouse bone marrow is compartmentalized into essentially self-contained stem cell regulatory volumes or domains. We estimate that W/W^v marrow contains about 2,600 stem cell regulatory units with an average volume of about 10^6 μm^3, a dimension consistent with the presumptive role of short-range cell-cell interactions in the regulation of pluripotent stem cells. Our analysis of the dose-response data is also indicative of the discontinuous and limited nature of local stem cell migration in a cellular marrow, a consideration that may be of practical as well as theoretical interest.

Received for publication 17 October 1977.

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IN VITRO ANALYSIS OF ALLOGENEIC LYMPHOCYTE INTERACTION

II. *I*-Region Control of the Activity of a B-Cell-Derived *H-2*-Restricted Allogeneic Effect Factor and its Receptor during B-Cell Activation*

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On the basis of studies with immunoadsorbent columns, many soluble, lymphocyte-derived, helper and suppressor factors, which mediate T-cell B-cell macrophage interaction during antibody synthesis, have been shown to consist of *H-2*-linked *I*-region associated (Ia)[1] antigens (1–3). It has been postulated that *I*-region genes also determine the putative receptors for both helper (4–6) and suppressor (7–10) factors. Both helper factors and their receptors have been reported to be controlled by the *I-A* subregion (4, 5, 10). Suppressor cell factors and their receptors have been found to be products of either the *I-J* subregion (7, 10) or the *I-C* subregion (9).

We have previously demonstrated that an antigen-nonspecific helper factor, allogeneic effect factor (AEF), is comprised of Ia antigens derived from both the activated responder and irradiated stimulator spleen cells of a mixed lymphocyte culture reaction (MLR) (11). This AEF helped a primary and secondary antibody response of both T-cell-depleted responder spleen B cells and stimulator spleen B cells. Identity in the *I-A* and/or *I-B* subregions was required for the interaction between this AEF and primary (nonimmune) spleen B cells.

An AEF produced by Ia-negative activated responder cells and irradiated T-cell-depleted stimulator cells helped a secondary antibody response of T-cell-depleted stimulator B cells but not responder B cells (12). This genetically restricted AEF was shown to contain Ia antigens determined by the stimulator haplotype but not the responder haplotype. These observations indicated that restricted AEF is the product of a stimulator B cell and/or macrophage.

In this report, we show that restricted AEF is a B-cell-derived helper factor which consists in part of active Ia antigen components determined by the *I-A* subregion. We also demonstrate that restricted AEF possesses a target B-cell receptor which may also be controlled by the *I-A* subregion. Taken together, the data suggest that the *H-2* restriction in the activity of restricted AEF

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* Supported by the Medical Research Council of Canada grant MA 5729.

[1] *Abbreviations used in this paper:* AEF, allogeneic effect factor; DNP, 2,4-dinitrophenyl; FCS, fetal calf serum; I, immune response; Ia, *I*-region associated; KLH, keyhole limpet hemocyanin; MLR, mixed lymphocyte culture reaction; NSE, nonspecific esterase; PFC, plaque-forming cell; RBC, erythrocytes; TNP, 2,4,6-trinitrophenyl.

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TABLE I

H-2 Haplotype Origin of Strains Used

Strain	Haplotype	Region[*]								
			I							
		K	*A*	*B*	*J*	*E*	*C*	*S*	*G*	*D*
B10.A	*a*	*k*	*k*	*k*	*k*	*k*	*k*/*d*[‡]	*d*	*d*	*d*
B10.BR	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*k*
B10.S	*s*	*s*	*s*	*s*	*s*	*s*	*s*	*s*	*s*	*s*
A.TL	*t1*	*s*	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*d*
A.TH, B10.S(7R)	*t2*	*s*	*s*	*s*	*s*	*s*	*s*	*s*	*s*	*d*
B10.HTT	*t3*	*s*	*s*	*s*	*s*	*k*	*k*	*k*	*k*	*d*
B10.S(9R)	*t4*	*s*	*s*	?	*k*	*k*	*d*	*d*	*d*	*d*
A.TFR5	*ap5*	*f*	*f*	*f*	*f*	*f*	*k*	*k*	*k*	*d*

[*] Haplotype origin of *H-2* regions according to references 21 and 39.

[‡] It is not yet certain whether the *I-C* subregion of the $H\text{-}2^a$ haplotype is derived from the $H\text{-}2^k$ or $H\text{-}2^d$ haplotype.

during B-cell activation is mediated, in part, by a recognition of complementarity between Ia antigens in this factor and *I*-region products in its receptor. A preliminary report of some of the data included here has been previously published (13).

Materials and Methods

Mice. All inbred strains of mice used in this study were maintained in our colony at the University of Toronto. These strains were derived from breeding pairs kindly supplied by Doctors H. O. McDevitt, Stanford University, Stanford, Calif., and D. C. Schreffler, Washington University, St. Louis, Mo.

Antisera. The antisera used in these studies and the respective *H-2* regions or subregions immunized against are as follows: A.TH anti-A.TL (anti-I^k, S^k, G^k); A.TL anti-A.TH (anti-I^s, S^s, G^s); (B10.A × A.TL)F_1 anti-B10.S(9R) (anti-$I\text{-}A^s$, $I\text{-}B^s$?); (B10.A × A.TL)F_1 anti-B10.HTT (anti-$I\text{-}A^s$, $I\text{-}B^s$, $I\text{-}J^s$) B10.HTT anti-B10.S(7R) (anti-$I\text{-}E^s$, $I\text{-}C^s$, S^s, G^s); and (B10.HTT × A.TFR5)F_1 anti-A.TH (anti-$I\text{-}E^s$, $I\text{-}C^s$, S^s, G^s). The latter three sera were a kind gift from Doctors D. B. Murphy and H. O. McDevitt, Stanford University, Stanford, Calif. All of the above sera were raised by hyperimmunization of recipient mice with donor spleen and lymph node lymphocytes as previously described (14). An AKR/J anti-AKR/Cum anti-Thy-1.2 serum, produced by using donor thymocytes and spleen cells for immunization, was used to deplete spleen cell suspensions of T cells. The *H-2* haplotypes of some of the strains used in this study are shown in Table I.

Antigens and Immunizations. The preparation of the 2,4-dinitrophenyl (DNP) keyhole limpet hemocyanin (KLH; Calbiochem, Downsview, Ontario) conjugate, DNP_{11}-KLH (per 10^5 daltons), and the immunization of mice with this antigen were performed as previously reported (12). Mice were sacrificed 4–8 wk postimmunization and were used as primed spleen cell donors.

Preparation of Restricted AEF. Restricted AEF was prepared as previously described (12). Briefly, as shown in Fig. 1, B10.BR($H\text{-}2^k$) lymphocytes were allogeneically activated against $H\text{-}2^s$ antigens by the intravenous injection of irradiated (800 rads) B10.S ($H\text{-}2^s$) recipients with 1–1.5 × 10^8 B10.BR donor thymocytes. 5 days later, the activated responder B10.BR cells were harvested from the stimulator B10.S recipients. Ia-negative responder cells were prepared by treatment of 10^8 cells with 1 ml of A.TH anti-A.TL diluted 1:5 for 20 min at room temperature. Cells were then centrifuged and resuspended in 3 ml of agarose-adsorbed rabbit complement diluted 1:9 and incubated for a further 45 min at 37°C, centrifuged, washed twice, and resuspended in serum-free Click's medium (15) supplemented with 4 mM glutamine and 5 × 10^{-5} M 2-mercaptoethanol before

Production of Restricted AEF

In Vivo Activation

B10.BR (H-2^k) Thymocytes (10^8) — Donor
↓ i.v. injection
B10.s (H-2^s) (irradiated with 800 rads) — Recipient
↓ 5 days
Recipient spleen cells removed (~90% of cells = donor T cells) — Activated Responder T cells

In Vitro Activation

Activated Responder T Cells (B10.BR)
–treated with anti Ia^k and complement

Mixed with

Irradiated Stimulator Cells (B10.S)

–treated with anti-Thy 1.2 and complement

FIG. 1. Production of restricted AEF.

culture. T-cell-depleted (<3% T cells, [12]) normal stimulator B10.S cells were similarly prepared by treatment with anti-Thy-1.2 (AKR/J anti-AKR/Cum) and rabbit complement. Restricted AEF was prepared by collection of supernates from 16–24 h MLR cultures between 10^7 Ia-negative activated responder B10.BR cells and 10^7 normal irradiated (3,000 rads) T-cell-depleted stimulator B10.S spleen cells. Culture conditions and medium were the same as reported earlier (12).

Immunoadsorption and Assay of Restricted AEF Activity. The methods used for the immunoadsorption on antibody-coated columns and assay of restricted AEF activity were previously described (12). The adsorption of restricted AEF activity was carried out during a 1 h incubation at 4°C on immunoadsorbents each consisting of approximately 5 mg of the IgG fraction a given mouse anti-Ia serum coupled to 1 g of cyanogen-bromide-activated Sepharose 4B (Pharmacia Fine Chemicals Inc., Dorval, Quebec). The activities of various column effluent fractions were then tested.

The cellular expression of a receptor for restricted AEF was analyzed by adsorption (9) of its activity by various cell types. All cell suspensions were prepared in serum-free Click's medium and were washed twice with this medium before adsorption. They were shown to contain greater than 90% viable cells as judged by trypan blue dye exclusion. The adsorbing cells used, derived from normal unprimed B10.S donors, were thymocytes, lymph node cells, spleen cells, spleen T cells prepared by passage through a nylon wool column (16), T-cell-depleted spleen cells prepared by treatment with anti-Thy-1.2 plus complement, Ia-negative T-cell-depleted spleen cells, and macrophage-depleted T-cell-depleted spleen cells (prepared as outlined in the section below). To prepare Ia-negative T-cell-depleted spleen cells, approximately 7.5 × 10^7 T-cell-depleted cells were washed twice, fractionated on a 1 g fetal calf serum (FCS) gradient to remove dead cells (17), washed twice in serum-free medium, treated with anti-Ia serum plus complement (see above), washed twice, and then used for adsorption. Approximately 10^7 cells were recovered from the original T-cell-depleted spleen cell population after this treatment. 1 ml of restricted AEF was incubated in undiluted form with 0.1–1.0 × 10^8 adsorbing cells in serum-free Click's medium at 4°C for 30 min with occasional mixing. After centrifugation of the cells, the activity of nonadsorbed AEF in the supernates was tested.

The helper activity of either unadsorbed or adsorbed (as above) restricted AEF was assayed in an in vitro secondary anti-DNP plaque-forming cell (PFC) response of DNP_{11}-KLH primed T-cell-depleted B10.S spleen cells after a 6 day culture in Click's medium containing 5% heat-inactivated FCS (Click-FCS). Anti-DNP indirect (IgG) PFC were evaluated in triplicate 0.3-ml cultures, containing 10^6 spleen cells, by the Cunningham and Szenberg method (18) by using 2,4,6-trinitrophenyl (TNP)-coupled burro erythrocytes (RBC) as indicator cells.

Depletion of Macrophages from Spleen Cell Suspensions. The phagocytic cells present in a T-cell-depleted spleen cell population were removed by the carbonyl-iron technique as previously reported (19). To a suspension of cells at 20 × 10^6/ml in Click-FCS was added powdered iron (Fisher Scientific Co., Don Mills, Ontario) at a concentration of 50 mg/ml. This suspension was incubated for 1 min at 37°C, then layered over an equal vol (5 ml) of Ficoll-Hypaque (Pharmacia Fine Chemicals, Inc., Dorval, Quebec and Winthrop Laboratories, Aurora, Ontario) and centrifuged at 450 *g* for 45 min. The cells banding at the Click-Ficoll interface were aspirated with a Pasteur pipette and washed twice with serum-free Click's medium before use. The extent of contamination with monocytes and/or granulocytes was determined cytochemically by the nonspecific esterase (NSE) staining method of Yam et al. (20).

Application of this technique to T-depleted spleen cells reduced the percentage of NSE-stained cells from approximately 5% to less than 1%. Thus, the technique was adopted to remove macrophages from T-depleted spleen cells used for the stimulator cell population in an MLR to generate restricted AEF and for the cellular adsorption of restricted AEF activity.

Results

I-Region Control of Restricted AEF Activity. By the use of appropriate immunoadsorbents, restricted AEF was previously shown to consist of Ia antigens derived only from the stimulator (H-2^s) and not responder (H-2^k) haplotype (12). The anti-Ia antiserum used for adsorption was A.TL anti-A.TH (anti-I^s, S^s, G^s), which potentially contains antibody activity against determinants of the entire *I*-region. It was therefore of interest to ascertain whether a specific I^s-subregion (s) codes for the Ia antigens present in restricted AEF.

Restricted AEF was fractionated on immunoadsorbents conjugated with either A.TL anti-A.TH (anti-I^s, S^s, G^s), (B10.A × A.TL)F$_1$ anti-B10.S(9R) (anti-$I\text{-}A^s$, $I\text{-}B^s$?), (B10.A × A.TL)F$_1$ anti-B10.HTT (anti-$I\text{-}A^s$, $I\text{-}B^s$, $I\text{-}J^s$), B10.HTT anti-B10.S(7R) (anti-$I\text{-}E^s$, $I\text{-}C^s$, S^s, G^s), or (B10.HTT × A.TFR5)F$_1$ anti-A.TH (anti-$I\text{-}E^s$, $I\text{-}C^s$, S^s, G^s). The activity of the column effluents of AEF was tested at a final concentration of 25% in a secondary anti-DNP PFC response of B10.S primed spleen B cells. Both this concentration and a 50% concentration of restricted AEF yield an optimum response under the conditions used here. Fig. 2 shows that AEF activity was adsorbed by antisera reactive with determinants of either the entire I^s subregion or only the $I\text{-}A^s$ and $I\text{-}B^s$ subregions and not the $I\text{-}E^s$ and $I\text{-}C^s$ subregions. The data suggest that restricted AEF Ia antigens are determined by the *I-A* and/or *I-B* subregion. However, it should be noted that the failure of the anti-$I\text{-}E^s$, $I\text{-}C^s$, S^s, G^s sera to adsorb restricted AEF activity may be accounted for by their rather weak cytotoxic antibody activity against B10.S lymph node target cells (approximately 15% specific cytotoxicity, titer = 1:10). By contrast, about 50–60% of B10.S lymph node target cells were specifically lysed by the sera containing anti-$I\text{-}A^s$, $I\text{-}B^s$? (titer = 1:320) and anti-$I\text{-}A^s$, $I\text{-}B^s$, $I\text{-}J^s$ (titer = 1:160) reactivity (T. L. Delovitch, unpublished observations). Accordingly, the control of restricted AEF Ia antigens by the *I-E* and *I-C* subregions may not be ruled out.

B-Cell Origin of Restricted AEF. We have previously suggested that restricted AEF Ia antigens are the products of a B cell and/or macrophage. However, the precise cellular origin of restricted AEF was not identified. Therefore, we have now analyzed the activity of a restricted AEF derived from Ia-negative responder cells and irradiated, T-cell-depleted, macrophage-depleted stimulator spleen cells. This stimulator cell population contained less

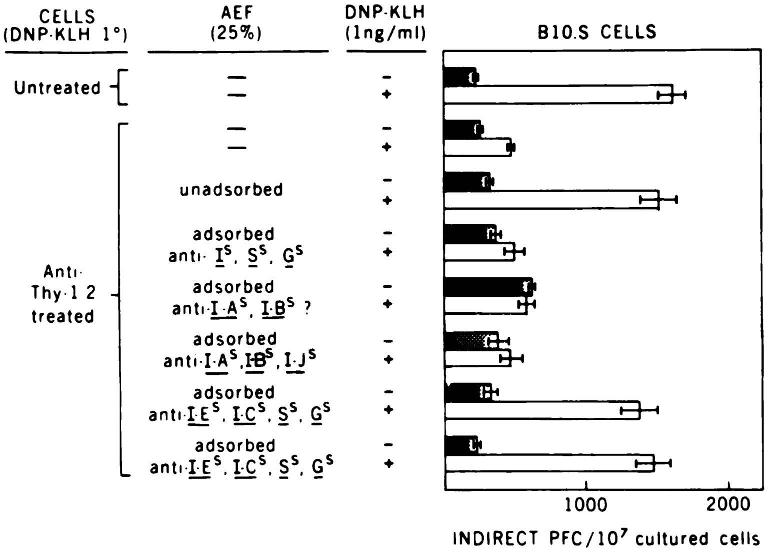

FIG. 2. Mapping of restricted AEF activity. Untreated and anti-Thy-1.2-treated DNP-KLH primed B10.S spleen cells were cultured with (+) or without (−) DNP-KLH (1 ng/ml) in the absence or presence of either unadsorbed or antibody column adsorbed restricted AEF (25% final concentration). The antisera and method used for the preparation of the immunoadsorbents are described in Materials and Methods. Indirect PFC in triplicate 6-day cultures in Click-FCS medium were enumerated by using TNP-coupled burro RBC as indicator cells. Direct PFC values ranged from 100 to 200/10^7 cultured cells and are not shown here. The results obtained from two experiments are presented as standard errors of the geometric mean.

than 1% esterase-positive cells and was thus considered to be essentially devoid of macrophages (see Materials and Methods). Fig. 3 shows that such a restricted AEF, when tested at a concentration of 50%, helped a secondary anti-DNP response of hapten-primed, macrophage-depleted T-cell-depleted spleen cells of stimulator B10.S origin but not responder B10.BR origin.

Thus, this restricted AEF displays a haplotype preference identical to that obtained by using Ia-negative responder cells and irradiated, T-cell-depleted stimulator spleen cells (12). More significantly, the presence of macrophages in the stimulator cell population does not seem to be required for the production of an active AEF. This result, taken together with the data presented in Fig. 2, suggests that restricted AEF Ia antigens are B-cell-derived products of the *I-A* and possibly *I-B* subregions.

I-Region Control of a Receptor for Restricted AEF. Genetic analysis of the activity of several factors with their appropriate target cells, derived from various *I*-region recombinant-inbred strains, has previously demonstrated a requirement for *I*-subregion identity between the factor-producing and target cell strains (4–9). In this study, we examined the ability of a restricted AEF, produced as mentioned in the previous section, to help B cells from various strains in a secondary response (Fig. 3). This AEF helped not only B10.S B cells, but also B10.HTT, B10.S(9R) and A.TH B cells. No response was obtained with A.TL B cells.

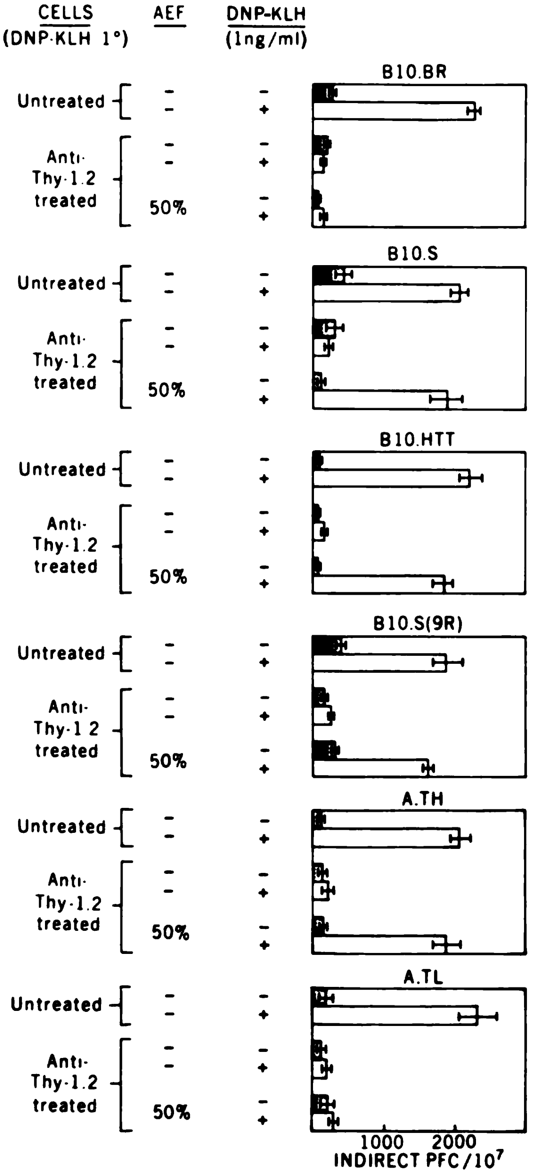

FIG. 3. Strain distribution analysis of restricted AEF activity. The activity of restricted AEF was assayed at a 50% concentration with primed, macrophage-depleted, anti-Thy-1.2-treated cells of various strains. The responses of untreated primed cells were included as controls. Culture conditions and evaluation of PFC are as in Fig. 2. The results of three experiments are shown.

Thus, restricted AEF activity is dependent upon *I*-region identity between e AEF producing strain (B10.S) and its selected target cell strain. The data lso suggest that macrophage-depleted, T-cell-depleted hapten-primed spleen lls derived from B10.S, B10.HTT, B10.S(9R), and A.TH express a receptor for stricted AEF. By reference to the *H-2* haplotypes of origin of the strains tested

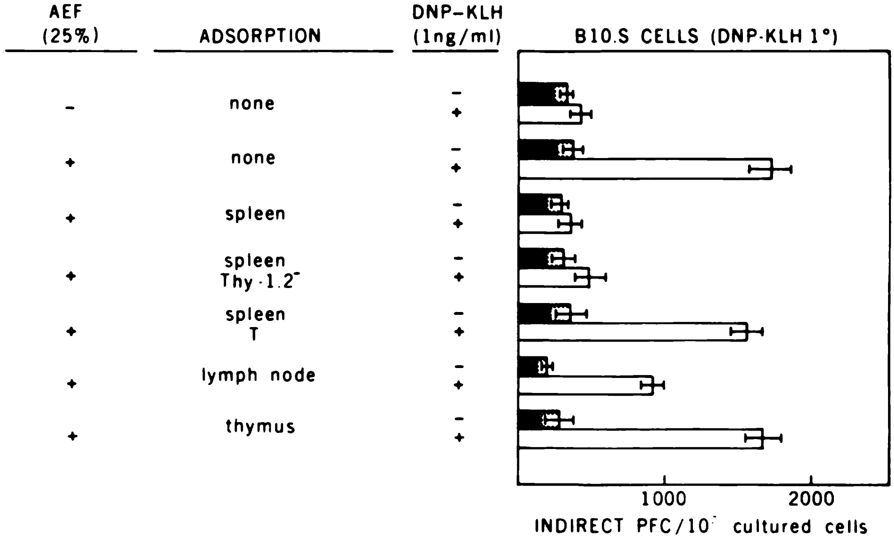

FIG. 4. Cellular adsorption of restricted AEF activity. The activity of restricted AEF was assayed at a concentration of 25% with DNP-KLH primed anti-Thy-1.2-treated B10.S spleen cells either before or after adsorption. 10^8 normal B10.S spleen cells, spleen Thy-1.2$^-$ (T-cell depleted) cells, spleen T cells (nylon wool nonadherent), lymph node cells, and thymus cells were used for adsorption. Culture conditions, number of experiments, and evaluation of PFC are as in Fig. 3.

(Table I), it is evident that this putative receptor is controlled by *H-2*-linked genes which map to the left of the *I-J* subregion. The result obtained with A.TL cells implies that the *K*-region is not involved here. It may be concluded, therefore, that the receptor for restricted AEF is a product of the *I-A* and/or *I-B* subregion.

Cellular Expression of a Receptor for Restricted AEF. The observation of cellular adsorption of the activity of a soluble lymphocyte-derived MLR suppressor factor has suggested the presence of a receptor for this factor on an activated T lymphocyte (9). The above strain distribution analysis of restricted AEF activity suggested that hapten-primed, macrophage-depleted, T-cell-depleted spleen B cells of several strains express a receptor for restricted AEF. However, no indication of whether this receptor can be found on T cells and macrophages, in addition to B cells, was presented. We therefore used the cellular adsorption technique in an attempt to identify the cell type(s) which expresses a receptor for restricted AEF.

Preliminary experiments demonstrated that restricted AEF activity was completely removed by adsorption on B10.S T-cell-depleted spleen cells obtained from either antigen-primed or normal (nonimmune) donors. Since normal donors were available in larger amount and also more frequently, all further adsorption studies were conducted with cells from normal B10.S mice. These experiments were performed with a restricted AEF prepared as described in Fig. 1. Fig. 4 shows that 10^8 spleen cells, T-cell-depleted spleen cells, and lymph node cells each adsorbed AEF activity. Complete adsorption was achieved by the spleen cell populations, while approximately only 50% adsorption was obtained with lymph node cells. These results may be explained by the relative number of B cells in these cell populations. It should be noted that a minimum of approximately 5 × 10^7 B-cell-enriched T-depleted spleen cells were required for complete adsorption (T. Delovitch, J. Biggin, and F-Y. Fung, unpublished observation). By contrast, 10^8 nylon wool-purified spleen T cells (2% Ig-positive) and thymocytes showed little or no adsorbing capacity. Prior activation of the latter two cell populations with concanavalin A failed to result in their adsorption of AEF activity (data not shown). Similar data were obtained from these various cell suspensions prepared from nonimmune B10.HTT donors but not from B10.BR or B10 donors (T. Delovitch, J. Biggin, and F-Y. Fung, unpublished observations).

These studies, along with those presented in Fig. 3, indicate that restricted AEF potentiates an antibody response of B cells derived only from strains which possess a receptor for this helper factor. They also imply that B cells, and perhaps macrophages, bear this receptor. In addition, it may be argued that some residual T cells in the T-cell-depleted spleen cell population adsorbed AEF activity and therefore also bear this receptor. However, this is unlikely for two reasons. First, this cell suspension contained less than 3% Thy-1.2-positive cells, as assayed by cytotoxicity by using the same AKR/J anti-AKR/Cum anti-Thy-1.2 serum originally employed for the depletion of T cells. Second, as noted above, no adsorption was observed with 10^8 nylon wool purified spleen cells.

To further examine the cellular origin of this receptor, a comparison was made between the adsorbing capacity of B10.S T-cell-depleted spleen cells and macrophage-depleted, T-cell-depleted spleen cells. 10^8 T-cell-depleted spleen cells and 8 × 10^7 macrophage-depleted, T-cell-depleted spleen cells, which were recovered after removal of phagocytic cells from the original 10^8 T-cell-depleted cells in the suspension, were used. As may be seen in Fig. 5, both these cell populations adsorbed virtually all of the activity of restricted AEF. Removal of macrophages from a T-cell-depleted spleen cell suspension does not reduce the adsorption capacity of the latter cell population. Moreover, no adsorption was obtained with 9 × 10^7 splenic adherent cells (adherent to plastic, see reference 6 for details), while an equivalent number of splenic non-adherent cells removed all AEF activity (data not shown). Hence, it is clear that B cells, but neither macrophages nor T cells, bear a receptor for AEF. It is also evident from Fig. 5 that the adsorbing capacity of T-cell-depleted spleen cells may be eliminated by pretreatment of this cell suspension with either A.TL anti-A.TH (anti-I^s, S^s, G^s) or (B10.A × A.TL)F_1 anti-B10.S(9R) (anti-$I\text{-}A^s$, $I\text{-}B^s$?) and complement. In each instance, only about 10^7 of the original 10^8 cells in the T-cell-depleted spleen cell suspension survived treatment with these sera. These findings therefore demonstrate that those B cells which possess a receptor for restricted AEF also bear surface Ia antigens. Furthermore, since nonimmune B cells were used for these adsorption studies, B cells need not be activated by antigen to induce the expression of this receptor.

Discussion

Further evidence is provided here for a regulatory role of Ia antigens in the activation of B cells to IgG antibody production. Ia antigens determined by the stimulator haplotype have been shown to be active components of restricted AEF (12). The control of these Ia determinants has now been localized to the *I*-

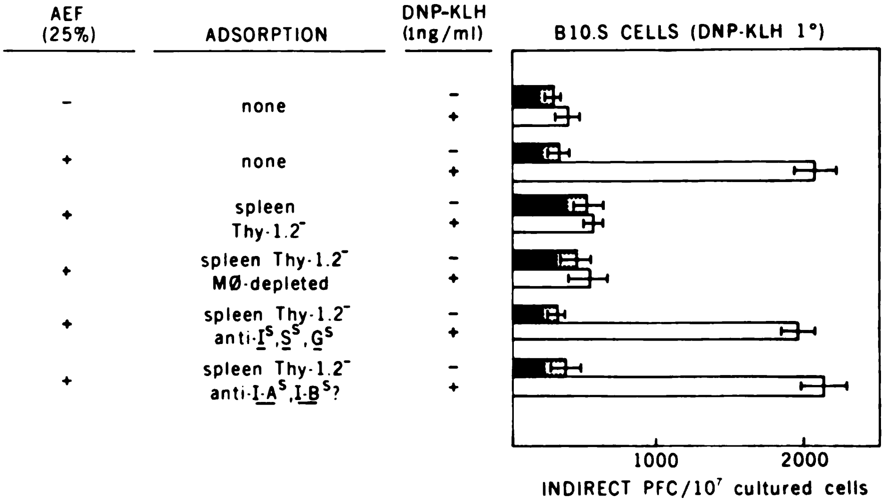

Fig. 5. Expression of a receptor for restricted AEF by Ia-positive spleen B cells. The activity of restricted AEF was assayed as in Fig. 4 at a concentration of 25% after adsorption on either 10^8 spleen Thy-1.2⁻ (T-cell-depleted) cells, 8 × 10^7 macrophage (mϕ)-depleted spleen Thy-1.2⁻ cells, or 10^7 spleen Thy-1.2⁻ cells that survived lysis by either A.TL anti-A.TH(anti-*I*^{s}, *S*^{s}, *G*^{s}) or (B10.A × A.TL)F$_1$ anti-B10.S(9R) (anti-*I-A*^{s}, *I-B*^{s}?) plus complement (Ia-negative, T-cell-depleted cells). Culture conditions, number of experiments and evaluation of PFC are as in Fig. 3.

A and/or *I-B* subregions. It is possible that the *I-B* subregion may not be involved here. Since the *H-2* haplotype origin of the *I-B* subregion of B10.S(9R) has not yet been identified (21), it is not certain whether the (B10.A × A.TL)F$_1$ anti-B10.S(9R) (anti-*I-A*^{s}, *I-B*^{s}?) serum used to adsorb restricted AEF activity detects a product of the *I-B*^{s} subregion. Moreover, none of the known Ia specificities have been mapped to the *I-B*^{s} subregion (21). An antibody which reacts with only an *I-A*^{s} subregion product may therefore be sufficient to completely adsorb this activity. It should also be cautioned, however, that additional control here by the *I-E*^{s} and *I-C*^{s} subregions cannot yet be excluded. No Ia specificities have as yet been localized to these *I* subregions (21). Several investigators have therefore found it difficult to produce high-titered cytotoxic antibody reactive with products of these particular *I* subregions. Consequently, the latter findings may explain why the sera containing potential anti-*I-E*^{s}, *I-C*^{s} reactivity did not adsorb any restricted AEF activity. While these mapping data are presently somewhat inconclusive, the findings that restricted AEF is definitely a product of the *I-A* subregion is compatible with reports that several other helper factors are also determined by this subregion (4, 5, 10).

Data presented here indicate that restricted AEF may be a B-cell product. The depletion of macrophages from the stimulator cell population used to produce restricted AEF does not reduce AEF helper activity. Similarly, Eshhar et al. (22) found that the removal of adherent cells from either the responder cells or the stimulator cells does not abrogate any of the helper activity of a nonrestricted AEF. Moreover, these investigators reported that this nonrestricted AEF possesses components derived from the stimulator cell population, as well as the responder cell population. This observation confirmed our previous findings (12). It may, therefore be concluded that macrophages do not produce AEF, nor are they required to be present for its production. These conclusions agree with those previously reported for a T cell replacing factor present in an allogeneic supernate (23). Nevertheless, it should be cautioned that the T-cell-depleted, macrophage-depleted, stimulator cell population used here to generate restricted AEF may still contain a nondetectable number of functional macrophages. In this report, macrophage depletion was assayed solely by the ability to remove esterase-positive cells. However, a small subpopulation of esterase-negative macrophages which readily phagocytose latex particles can mediate a primary IgM anti-hapten PFC response (C. Cowing, Immunology Branch, National Institutes of Health, personal communication). It is not known whether this macrophage subpopulation bears Ia antigens. Macrophages are known to be Ia positive (17), relatively radiation-resistant (6), and stimulatory in an MLR (24). Thus, it is conceivable that Ia antigens in AEF are derived either wholly or in part from esterase-negative ($<1\%$ esterase-positive), irradiated, stimulator splenic macrophages. If this is the case, a possible macrophage origin for AEF must then be considered.

MLR responder T cells have been purported to be Ia negative because pretreatment of these cells with anti-Ia serum plus complement does not inhibit their subsequent MLR response (11, 25). Other studies have shown by immunofluorescence that the Ly-1^+, 23^- subpopulation of MLR responder T blasts, during activation across an *I*-region incompatibility, can bind Ia antigens derived from the stimulator haplotype (26). It has also been demonstrated that, during an MLR, the Ly-1^+, 23^- subpopulation of responder T cells recognizes allogeneic Ia antigens on stimulator cells (27). Consistent with this finding is the report that the elimination of Ly-1^+, 23^- T cells from the activated responder cells used in the generation of a nonrestricted AEF results in the loss of about 85% of AEF helper activity (22). Hence, during the in vitro production of restricted AEF, Ia antigens determined by the stimulator haplotype may be secreted by the stimulator B cell and may then bind in a specific fashion to an Ia-negative responder T cell. This responder T cell would now become Ia positive and may then release these Ia antigens, perhaps in combination with another Ia-negative component(s), into AEF. If this sequence of events were to occur, AEF Ia antigens would then have to be considered to be B-cell-derived, T-cell-dependent, products. This conclusion would support our hypothesis that AEF does indeed have a B-cell origin. It would also support our previous suggestion that Ia antigens found on T-cell membranes are passively adsorbed products synthesized by B cells (17). AEF would therefore represent the first Ia-positive helper factor to be identified as being a B-cell product. This line of reasoning would also be compatible with a previous claim that AEF is T-cell derived (28). Thus, attempts will be made in the future to determine whether Ia antigens controlled by the stimulator haplotype appear on the surface of either untreated or anti-Ia plus complement-treated MLR activated responder T cells used to produce AEF.

A strain distribution analysis of the capacity of restricted AEF to stimulate a secondary anti-DNP PFC response confirmed a previous expectation (12), that this factor can help B cells of only its own haplotype, or of haplotypes which express identical Ia antigens. An *I-A* and/or *I-B* subregion identity between the restricted AEF producing strain and the target cell strain was required for this response. The responses obtained here are consistent with previous reports that the stimulation of an antibody response by a positive allogeneic effect involves a cooperative recognition of *I*-region products (29). By contrast, *I*-region products are not recognized during a negative allogeneic effect which results from the suppression of an immune response by allogeneic T cells (27, 29).

Thus, the *I*-subregion determinants which are present in restricted AEF must also appear on the surface of its target cell. This suggests that the target cell bears a specific receptor for restricted AEF and that this receptor and restricted AEF are both products of the same *I*-subregion(s). Since it has been tentatively concluded above that restricted AEF Ia molecules are determined by *I-A*, this *I*-subregion may also control the receptor postulated to exist here. The suggestion that AEF and its receptor are encoded by the *I-A* subregion would agree closely with the previously reported control by this *I*-subregion of other helper factors and their receptors (4–6, 10).

Studies performed here demonstrate that the B cell represents the target cell of AEF. Furthermore, they suggest that an Ia-positive B cell expresses a receptor for AEF. It appears that neither a prior antigen-activation of the B cell nor a specific surface modulation event is necessary for the expression and function of this receptor. These data therefore strongly support the earlier evidence that AEF produced in the usual manner (12, 28) and other antigen-nonspecific T-cell replacing factors (30, 31) act directly on B cells during either a primary IgM or a secondary IgG antibody response. The latter factors described both stimulate a B-cell response which is macrophage independent.

T cells and macrophages from either unprimed or antigen-primed donors apparently do not express a receptor for restricted AEF, or otherwise possess a substantially lower density of receptors which is below the limit of sensitivity of detection achieved here. These results differ from those previously obtained with Ia-positive T-cell-derived suppressor factors and an Ia-positive macrophage-derived helper factor, which are purported to possess *I*-region determined receptors on antigen-activated T cells (7, 9) and normal T cells (6), respectively.

As mentioned above, several in vitro studies of IgG antibody synthesis have shown a requirement for *I*-region compatibility between several helper factors and their respective receptors. Similarly, *I*-region identity is required for effective T-cell B-cell interaction in vivo. Katz et al. (32) have reported that antigen-primed T and B cells must share an *I-A* subregion identity to cooperate for a secondary IgG antibody response. Press et al. (33) have demonstrated that a secondary IgG anti-DNP response may be mediated by a specific recognition by carrier-primed T cells of syngeneic Ia antigens on hapten-primed B cells. The same conclusion was reached by Pierce and Klinman (34) who showed that the transfer of primary DNP-specific B cells into allogeneic carrier-primed recipients resulted in only an IgM anti-DNP response; the transfer of either primary or secondary DNP-specific B cells into syngeneic carrier-primed recipients resulted in only an IgG response (35). In the latter studies, stimulation of IgG production was dependent on an identity in the *I-A* subregion between the collaborating T and B cells.

The apparent need for *I*-region compatibility exemplified in vitro in this study may therefore represent an event of normal B-cell activation during IgG antibody synthesis in vivo. It may be envisaged, as proposed above, that after antigenic stimulation a T cell recognizes Ia determinants on a B cell and then passively acquires these Ia molecules onto its surface. This Ia-positive T cell may then interact with a syngeneic antigen-presenting cell, i.e., a macrophage. Such an interaction might induce the release from the T cell of the immunizing antigen or fragment thereof, Ia antigens, and perhaps other membrane-associated components. These T-cell released components, which may or may not become associated in a complex structure, might be recognized and bind to their specific receptors on a B cell. Such a mechanism would allow for an *I*-region identity to be achieved at both the macrophage T cell and T cell B cell levels of interaction. It would also enable a T cell to recruit and collaborate with an Ia compatible B cell.

A two-signal hypothesis has been proposed for B-cell triggering (36, 37). Antigen represents the first signal that binds to its B-cell immunoglobulin receptor. A T-cell factor or thymus-independent antigen may act as a second signal and bind to another B-cell surface receptor. AEF, which consists of Ia antigens and perhaps additional Ia-negative components, may mediate T cell B cell collaboration by serving as a second signal given to the B cell during the stimulation of an antibody response. Hence, a specific recognition between an Ia molecule in AEF and a complementary component(s) in its B-cell receptor could conceivably occur during B-cell activation.

The immunochemical nature of the receptor for AEF is not known. It has been suggested here that this receptor is an *I*-region gene product. The possibility has also been raised that the receptor for another nonantigen-specific mediator of B-cell responses bears Ia determinants (38). However, it is also possible that these receptors are not comprised of Ia antigens. One alternative to be considered in the present study is that Ia antigens on the B cell may become associated with the receptor for AEF only after the formation of a factor receptor complex. The receptor may in fact be controlled by a non-*H-2*-linked gene. It may recognize an Ia-negative T-cell-derived component of AEF which is complexed to an Ia molecule. The *I*-region compatibility requirement discussed earlier may be fulfilled solely by the interaction of AEF Ia antigens and syngeneic Ia antigens on a nonreceptor portion of the B-cell membrane. These possibilities may only be analysed by a comparative biochemical analysis of this B-cell receptor (if it can be isolated), B-cell surface Ia antigens and Ia antigens in AEF. Such studies are currently in progress.

Summary

A genetically restricted allogeneic effect factor (AEF) derived from a mixed lymphocyte culture reaction between Ia-negative activated responder cells and irradiated T-cell-depleted stimulator cells was characterized. Restricted AEF is a B-cell-derived soluble helper factor which consists in part of Ia antigens controlled by the *I-A* subregion of the stimulator haplotype; additional control by the *I-B*, *I-E*, and *I-C* subregions, although unlikely, could not be excluded. This factor helps B cells of only its own haplotype or of haplotypes which carry an *I-A* and/or *I-B* subregion identity. Unprimed as well as hapten-primed Ia-positive B cells express a receptor for restricted AEF. The results indicate that the B-cell receptor for AEF is determined by the *I-A* subregion. Both restricted

AEF and its receptor may therefore be products of the same *I*-region gene(s). The data are compatible with the hypothesis that the AEF Ia antigens serve as a second signal required for B-cell activation to IgG antibody production.

We thank Doctors A. Cunningham, E. Gelfand, and A. Marks for their helpful suggestions in the preparation of this manuscript. We also thank Ms. Patricia Camden for her fine secretarial assistance.

Received for publication 20 October 1977.

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EFFECT OF COLCHICINE ON THE ANTIBODY RESPONSE

I. Enhancement of Antibody Formation in Mice*

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In 1954, some experiments were carried out in this laboratory on the effect of colchicine (CC)[1] on antibody formation in rabbits. At that time, we had found that antibody formation was associated with rapid and extensive cell divisions in the plasma cell and its immediate precursors which had just been associated with antibody synthesis (1). We thought, therefore, that the injection of CC would inhibit antibody formation, and Tanaka and Coons carried out some experiments to investigate this proposition. Much to our surprise, CC enhanced the antibody response in rabbits by a factor of ≅ 8. The results of these experiments are simple to relate. We administered antigen to naive or primed rabbits with or without CC, and measured the antibody response in the serum. The enhancing effect was found to depend on the administration of CC on the same day as the antigen. It was ineffective when given 2 days before or 2 days after the injection of antigen. Indeed, it had a slightly depressive effect when given 2 days after the antigen, and an even greater effect when given on day 4 after antigen administration. But given simultaneously with the antigen, it had an increasingly pronounced effect as the dose was increased from 0.5 to 2 mg/kg. The latter dose killed three of the four rabbits tested, but the survivor had an antibody titer on the 8th day of his response 50 times higher than the control rabbits which received no CC. These facts in the rabbit were true for both the primary and secondary antibody response. At that time, we were at a loss to explain these findings and published them only in an abbreviated form (2, 3).

When Gershon discovered the existence of the suppressor cell in 1970 (4), it seemed possible that the effect of CC could be explained by the elimination of some or all of the suppressor cells which are evidently stimulated to arise during every antibody response.

It is noteworthy that in 1952, Taliaferro et al. (5) investigated the effect of X-irradiation (total body radiation with 600 or 700 rads) on hemolysin production against sheep erythrocytes in the rabbit. They found that peak titers were reached when the antigen was administered from 6 h to 10 min before irradiation took place. 10 years later, Dixon and McConahey (6) also investigated the effects of whole body radiation on the antibody response to bovine gammaglobulin in the rabbit. They found that the peak antibody titer to a primary stimulus was about four times that of the control value and appeared when the irradiation was carried out 2.5 days after the injection of antigen, although there was also a smaller elevation when the irradiation was carried out 1 day after an antigen injection.

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* Supported by grant 20811 from the National Institutes of Health.

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[1] *Abbreviations used in this paper:* BSS, balanced salt solution; CC, colchicine; DT, diphtheria toxoid; HGG, human gamma globulin; KLH, keyhole limpet hemocyanin; LCC, lumicolchicine; LD_{50}, mean lethal dose; PFC, plaque-forming cells; SRBC, sheep erythrocytes; TKB, TNP-KLH-bentonite; TNP, 2,4,6-trinitrophenyl; VB, vinblastine.

Fourfold enhancement of diphtheria antitoxin in guinea pigs was described by Greenberg and Fleming (7) when pertussis vaccine was injected subcutaneously at the same time. Johnson et al. (8) described the enhancement of antibody formation to protein antigens by the injection of endotoxin from bacterial cell wall.

In 1963, White (9) published a review entitled "Factors affecting the antibody response" in which he described some unpublished data (Farthing and White, 1959) which demonstrated the enhancing effect of CC (1 mg/kg) on both the primary and secondary response of guinea pigs to diphtheria toxoid. They reported about a 10-fold difference in both primary and secondary responses when CC was administered 6 h before or 6 h after the antigen.

In 1976, Bash, Singer, and Waksman (10) reported that cyclophosphamide (20–50 mg/kg) given on the day of antigen injection to donor rats abrogated the suppressive effect on recipients of the T-cell fraction of rat spleen cells. Finally, Burchiel and Melmon (11) reported that the mitotic inhibitors, CC, cytosine arabinoside, and hydroxyurea, all produced dose-dependent augmentation of the antibody response in cultures of murine spleen cells.

These findings clearly predict the existence of a suppressor cell since both X-ray and CC were known to kill dividing cells. The effect of endotoxin is not so clear, but perhaps it interferes with cell division at a critical moment.

In view of our early findings with CC and the effects of X-ray and endotoxin, we decided to test the working hypothesis that these materials killed suppressor cells. To do this, it was necessary to carry out experiments with animals in which cell transfers could be carried out, namely inbred mice. This paper describes the conditions required for the effective enhancement of the antibody response by CC in mice. Our results indicate that CC is effective in promoting the antibody response of mice to protein antigens, e.g. diphtheria toxoid and human gamma globulin, as well as to a hapten, 2,4,6-trinitrophenyl (TNP). For maximal enhancement, the drug must be administered simultaneously with the antigen. In an accompanying paper, we shall present evidence which demonstrates the action of CC on suppressor cells.

Materials and Methods

Animals. Male or female BALB/c mice, 8- to 16-wk-old, were obtained from The Jackson Laboratory, Bar Harbor, Maine; from Charles River Breeding Laboratories, Wilmington, Mass.; or from the West Seneca Laboratory, Health Research Inc., West Seneca, N. Y. Animals were maintained in cages with free access to laboratory mouse chow and acidified, chlorinated water.

Antigens and Immunization. Purified diphtheria toxoid (DT) and human gamma globulin (HGG) were supplied by the Massachusetts Department of Public Health, Division of Biologic Laboratories, Boston, Mass. The hapten-carrier conjugate, trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH), was prepared from 2,4,6-trinitrobenzene sulfonic acid (Eastman Kodak Co., Rochester, N. Y.) and KLH (Calbiochem, San Diego, Calif.) according to the procedure described by Rittenberg and Amkraut (12). The conjugate used in the present study had a ratio of 762 mol of TNP/mol of KLH. In some experiments, TNP-KLH was absorbed on bentonite particles by the method of Gallily and Garvey (13) for the purpose of immunization. All mice were immunized intraperitoneally. The doses of antigens used for immunization are specified in the text. CC (Sigma Chemical Co., St. Louis, Mo.) in physiological saline was administered intraperitoneally to appropriate groups of animals at doses ranging from 0.25 to 1.5 mg/kg body weight, depending upon the experimental protocol.

Passive Hemagglutination. Mice were bled from the ophthalmic venous plexus and the sera obtained were inactivated at 56°C for 30 min. Circulating antibody levels were measured by the hemagglutination of sheep erythrocytes, (SRBC; Colorado Serum Co., Denver, Colo.) covalently coupled to the protein antigen by bis-diazobenzidine according to the method of Stavitsky and Arquilla (14). For the detection of hapten-specific antibodies, the indicator SRBC were coupled to

TNP as described by Rittenberg and Pratt (15). Antibody titers were expressed as the reciprocal of the highest serum dilution which gave positive hemagglutinating reactions.

Preparation of Cell Suspensions and Hemolytic Plaque Assay. Mice were sacrificed by cervical dislocation. The spleens were harvested and gently teased with sharp forceps in balanced salt solution (BSS; 16). The dispersed spleen cells were filtered through stainless steel screens and were washed three times in BSS by centrifugation at 1,000 rpm for 10 min each time. The washed spleen cells were assayed for antibody-forming cells by a modified method (16) of the Jerne hemolytic plaque technique (17). Indicator SRBC were prepared according to the method of Kapp and Ingraham (18) for HGG-SRBC and of Rittenberg and Pratt (15) for TNP-SRBC. Briefly, the assay of plaque-forming cells was performed as follows. Glass culture tubes (10 × 75 mm) containing 0.3 ml of 0.7% agarose (L'Industrie Biologique Francaise S. A., Gennevilliers, France) in BSS, 50 μl of HGG-SRBC (7.5%) and 20 μl of SRBC-absorbed 0.5% bovine serum albumin solution were preincubated in a 41-42°C water-bath. 50 μl of a spleen cell suspension containing 10^5-10^6 viable nucleated cells were added to each tube. The mixture was gently but thoroughly mixed and was then spread on a microscope glass slide previously coated with 0.1% agarose. After the agarose had solidified, the slides were inverted, placed on plexiglass trays and incubated at 37°C in a humidified chamber. After an incubation period of 1.5-2.0 h, freshly reconstituted guinea pig complement (Pel-Freez Farms, Inc., Rogers, Ark.), at 1:15 dilution, was flooded under the slides. After an additional 1.5 h incubation, the number of plaque-forming cells (PFC) on each slide was enumerated under a low-power microscope. For the development of IgG PFC, a previously determined optimal dilution of a polyvalent rabbit anti-mouse IgG antiserum (kindly supplied by Dr. Carl W. Pierce of the Jewish Hospital of St. Louis, St. Louis, Mo.) was incorporated in the agarose mixture. The number of IgG PFC was calculated from the difference between the number of IgM PFC and the total number of PFC developed with the anti-IgG antiserum.

Preparation of Lumicolchicine. Lumicolchicine was prepared by the irradiation of a CC solution in a quartz cuvet placed at 12 inches from a UV lamp (Osram High Pressure Mercury Arc, HBO 200; Osram, Munich, W. Germany) for 30 min. The successful conversion of CC to predominantly beta- and gamma-lumicolchicines was monitored by the appearance of two isosbestic points at 255 and 305 nm in their UV spectra, and by a decrease of absorbancy at 350 nm (19).

Results

Primary Antibody Response. Mice injected intraperitoneally with 100 μg of TNP-KLH-bentonite (TKB) on day 0 gave a peak hapten-specific IgM PFC response on days 6 and 7 (Fig. 1 A). The simultaneous administration of CC (1 mg/kg body weight) with 100 μg of TKB to animals in the experimental group increased the IgM PFC response by about twofold. The hapten-specific IgC PFC response of CC-treated animals was also greater than that of control animals (Fig. 1 B). Significant elevation in the circulating anti-TNP antibody titers was observed in immunized animals treated with CC (Fig. 2). The enhanced antibody level appeared as early as 7 days after immunization, and the difference in antibody titer between the two groups lasted for at least 14 days. The administration of CC to animals immunized with HGG also significantly ($P < .001$) enhanced the serum antibody titer at 1 wk, but not 4 days, after immunization (Fig. 3). DT was found to be a relatively poor antigen for the induction of a primary response in mice even when CC was given.

Secondary Antibody Response. The immunization of mice with two injections of DT (20 Lf per injection) 20 days apart, gave a consistent response in terms of the mean circulating antibody titer 10 days after the second injection of antigen (Fig. 4). When colchicine was also administered to similarly immunized animals at the time of priming (group II), at the time of challenge (group III), or at both times (group IV), the subsequent antibody response was increased by about 15-fold. The kinetics of the secondary response in mice which received

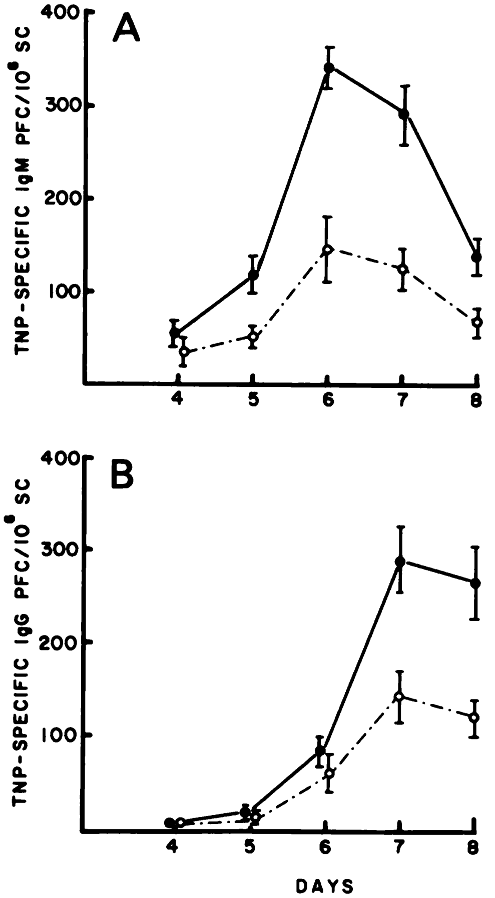

FIG. 1. Effect of CC on the kinetics of the TNP-specific primary PFC response. BALB/c mice were immunized intraperitoneally with 100 μg of TKB (○) or with 100 μg of TKB and CC (1 mg/kg, ●); their spleens were harvested for PFC assay at various times after immunization. Each point represents the mean ± SE of the response of 4-16 animals. SC, spleen cells.

two injections of DT and CC showed that the enhanced response occurred as early as 5 days after the second immunization (Fig. 5). The difference in antibody titers between CC-treated animals and control animals lasted for more than 15 days. The effective CC dose for maximal enhancement was between 1.0-1.5 mg/kg body weight (Fig. 6). The drug was found to be lethal to injected animals at doses above 1.5 mg/kg and the mean lethal dose (LD_{50}) of CC for BALB/c mice was 2.1 mg/kg (Fig. 7). Since a relatively large amount of purified DT would have been needed for the sensitization of indicator SRBC in a hemolytic plaque assay, the number of DT-specific antibody-forming cells was not enumerated. Instead, the effect of CC on the PFC response to another protein antigen (HGG), and to a hapten (TNP) was studied.

Mice previously primed with HGG and challenged 10 days later with the same antigen gave a relatively low HGG-specific IgG PFC response (Fig. 8 A). On the other hand, similarly immunized animals which were also given CC

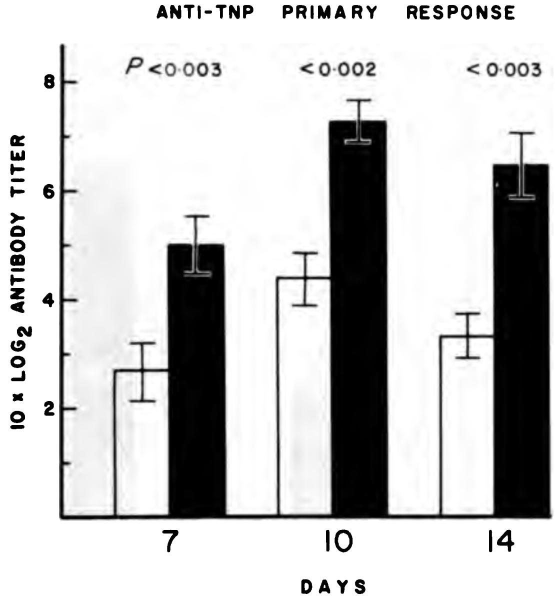

FIG. 2. CC-induced enhancement of serum anti-TNP hemagglutinating antibody levels. Mice were injected intraperitoneally with 100 μg of TKB (▭) or with 100 μg of TKB and CC (1 mg/kg, ■). Animals were bled on days 7, 10, and 14 after the injection. Each bar represents the mean titer ±SE of 6–7 animals.

showed an IgG PFC response which was about three times as large. Since very few, if any IgM PFC were detected by this system (20), we could only express our HGG-specific PFC response by the number of indirect plaques obtained. The increase in the number of antibody-forming cells was accompanied by elevated circulating antibody levels which persisted for a longer period of time (Fig. 8 B). In the case of the secondary PFC response to the TNP hapten, the administration of CC to experimental animals at the time of priming enhanced both the IgM and the IgG response (Fig. 9). The IgM PFC response of the CC-treated animals was twice as large (Fig. 9 A), and the IgC PFC response was about four times as large as that of the control animals (Fig. 9 B).

Relationship between the Time of Administration of CC and the Enhancement of the Antibody Response. Different groups of animals were given CC at times before (day −1), during (day 0), or after (day +1 and day +2) the injection of TNP-KLH. Animals in the control groups were injected with the antigen alone. The primed animals were challenged with TNP-KLH on day 14. 3 days after the second injection, the spleens of the immunized animals were assayed for their secondary hapten-specific PFC response. Maximal enhancement of the PFC response was observed when CC was administered to animals on the same day as the injection of antigen (Fig. 10 A). Although minimal enhancement was observed when the drug was given to animals 1 day before immunization, no elevation in the PFC response was noted when the drug was administered to animals 1 or 2 days after immunization. Similar results were observed in the primary response; the drug was most effective when given to animals on the same day as antigen injection (Fig. 10 B).

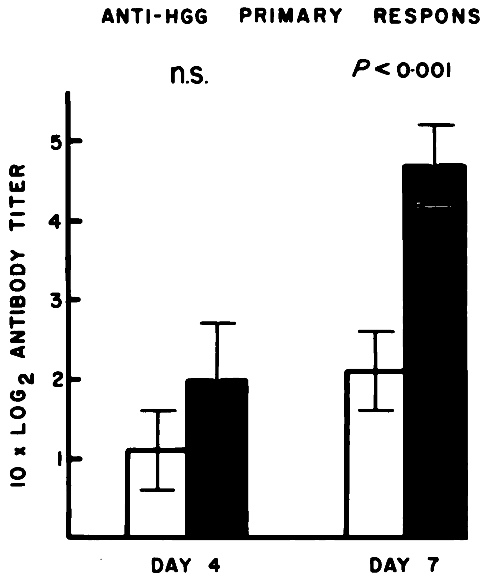

FIG. 3. Effect of CC on the circulating anti-HGG antibody titers. Mice were injected intraperitoneally with 100 μg of HGG (▭) or with 100 μg of HGG and CC (1 mg/kg, ■). Each bar represents the mean titer ± SE of 6 animals bled on day 4, and of 13 animals bled on day 7. n.s., not significant.

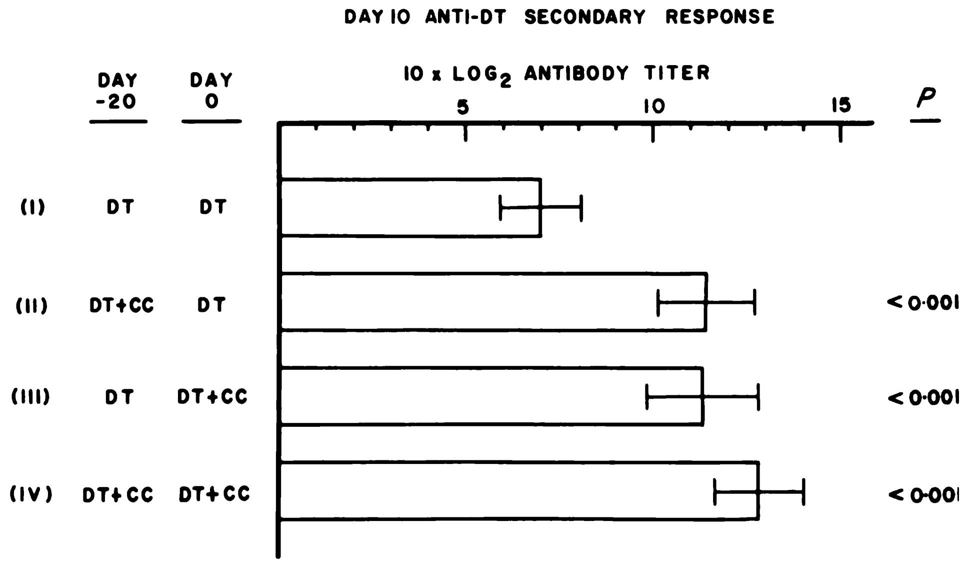

FIG. 4. CC-mediated enhancement of the secondary antibody response of mice immunized with DT. Control animals in group I were primed with 20 Lf of DT on day −20 and challenged with a similar dose of antigen on day 0. Animals in the experimental groups were immunized in a similar fashion except that CC, at a dose of 1 mg/kg body weight, was also administered at the time of priming (group II), at the time of challenge (group III), or at both times (group IV). All animals were bled 10 days after the second injection. Each bar represents the mean titer ± SE of 8 animals.

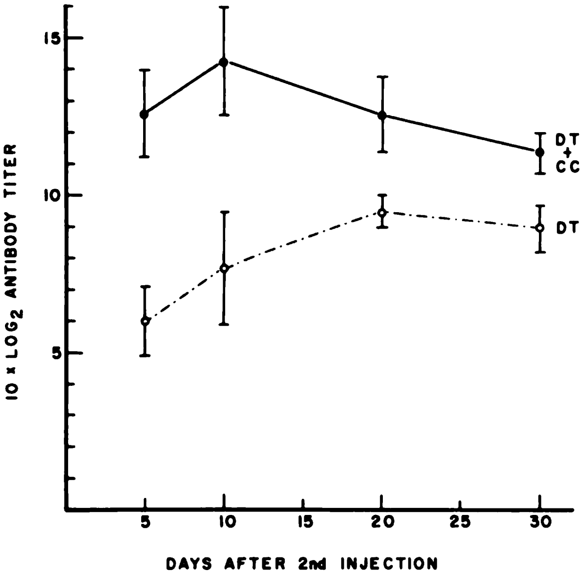

Fig. 5. Effect of CC on the kinetics of the secondary response to DT. Control animals (○) were given two intraperitoneal injections of 20 Lf of DT 20 days apart. Animals in the experimental group (●) were similarly immunized except that CC, at a dose of 1.5 mg/kg, was also administered at the time of antigen injection. Each point represents the mean titer ± SE of 7–8 animals.

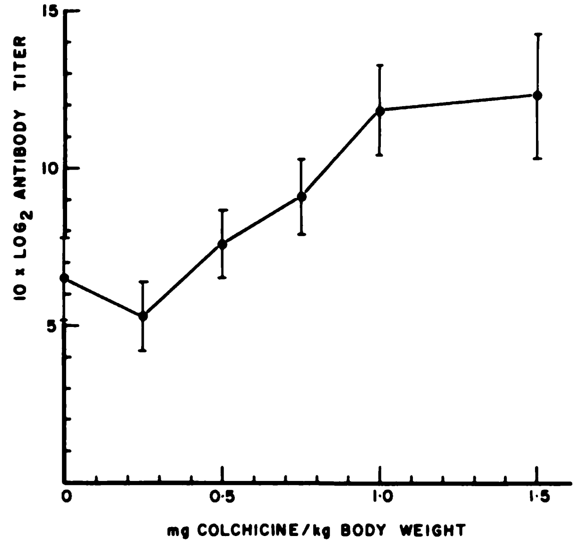

Fig. 6. Titration curve of the effect of different doses of CC on antibody formation. Control mice were given two intraperitoneal injections of 20 Lf of DT 20 days apart. Animals in the experimental groups were similarly immunized except that CC, at various doses as indicated in the abscissa, was also given. All animals were bled 10 days after the second injection, and their serum anti-DT hemagglutinating antibody titers were determined. Each point represents the mean titer ± SE of 5 animals.

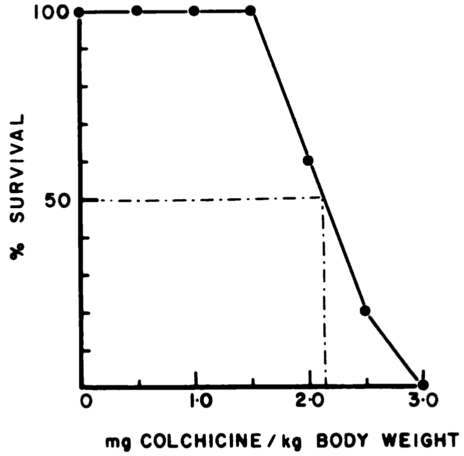

FIG. 7. Determination of the LD_{50} of CC in mice. BALB/c mice (10 animals/group) were injected intraperitoneally with various doses of CC ranging from 0 to 3.0 mg/kg body weight. Each point represents the percent of animals which survived for at least 10 days after CC injection.

Comparison of the Effect of Two Different Anti-Mitotic Drugs on the Antibody Response. Vinblastine (VB), another anti-mitotic drug, was tested for its effectiveness in enhancing the antibody response. Three groups of animals were similarly immunized with TKB. Animals in group II also received CC (1 mg/kg) and those in group III were injected with a similar dose of VB on the day of immunization. 6 days later, the spleens of the treated animals were assayed for their hapten-specific PFC response. Results shown in Table I indicate that VB was as effective as CC in enhancing the anti-TNP PFC response.

Effect of Lumicolchicine on the Antibody Response. Lumicolchicine (LCC), the structural isomer of CC, was tested for its effectiveness to enhance antibody formation. Mice in the control group were injected intraperitoneally with 100 μg of TKB on day 0. Two other groups of animals were immunized in a similar manner, except that CC was also administered to 1 group and LCC to the other. The dose given in each case was 1 mg/kg body weight. The hapten-specific PFC response was assayed on day 6. Results shown in Table II indicate that whereas CC enhanced the TNP-specific PFC response by more than 100%, the administration of LCC to immunized animals had no enhancing effect.

Discussion

Results of experiments performed in mice and reported in the present paper confirm the original observations by Tanaka and Coons (2) that CC is effective in enhancing the antibody response. Apparently, there is no species difference in terms of the capacity of CC to promote antibody formation since the drug works well in rabbits (2, 3), guinea pigs (9), hamsters (21), and in mice as presently reported.

Previous reports on CC-induced enhancement expressed the enhancing effect only in terms of the serum antibody level, and the magnitude of the maximal enhancement varied from 3- to 50-fold (2, 9, 21, 22). In the present study with

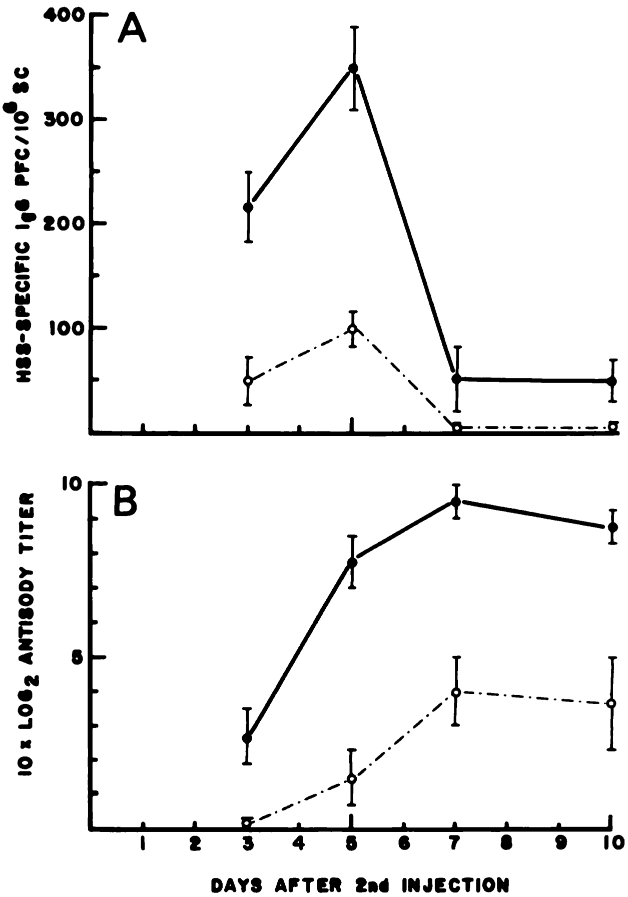

FIG. 8. Enhancement of the secondary antibody response to HGG by CC. Mice in the control group (○) were given 2 intraperitoneal injections of 100 μg of HGG 14 days apart. Animals in the experimental group (●) were similarly immunized except CC, at a dose of 1 mg/kg, was also administered at the time of antigen injection. At various times after the second injection, the animals were bled for the determination of serum hemagglutinating anti-HGG antibody titers (B) and their spleens were harvested for the assessment of their PFC response (A). Each point represents the mean response ± SE of 4-10 animals. SC, spleen cells.

mice, our data indicate that CC-induced enhancement of the circulating antibody titer varied from as low as 3-fold (in the primary response to HGG, Fig. 3) to as high as about 15-fold (in the secondary response to DT, Fig. 4) more than that of the control group. In addition to evaluating the serum antibody titer, this study also examined the cellular aspect through the assessment of the PFC response. The data indicate that the number of antibody-forming cells in the spleens of CC-treated animals was significantly increased. For example, the hapten-specific primary PFC response was doubled (Fig. 1) and the secondary IgG PFC response was enhanced by at least fivefold (Fig. 9). In the case of the secondary PFC response to HGG, the response of CC-treated animals was at least three times as large as that of control animals (Fig. 8 A). This

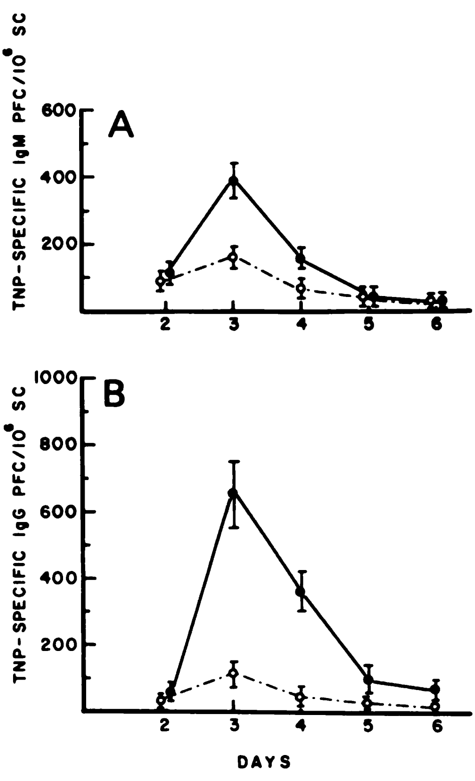

FIG. 9. Effect of CC on the secondary TNP-specific PFC response. Mice in the control group (O) were given 2 intraperitoneal injections of 100 μg of TNP-KLH 14 days apart. Animals in the experimental group (●) were similarly immunized except CC, at a dose of 1 mg/kg, was also given at the time of priming. On each day between 2 and 6 days after challenge, spleens of the immunized animals were harvested for the assay of their hapten-specific PFC response. Each point represents the mean response ± SE of five animals. SC, spleen cells.

increase in the PFC response was accompanied by an elevation of the serum antibody titer (Fig. 8 B). These results implied that the administration of CC at the time of antigenic penetration affected certain cellular events leading to an increase in the number of antibody-forming cells which in turn increased the circulating antibody level.

For effective enhancement of the antibody response by CC, there are at least two critical factors which have to be observed; (*a*) the dose of CC used, and (*b*) the time of its administration.

Within the dose range that is not lethal for mice, maximal enhancement may be obtained by the injection of 1.0–1.5 mg CC/kg body weight (Fig. 6). An increasingly pronounced enhancing effect was also observed in the rabbit when the dose of CC was increased from 0.5 to 2.0 mg/kg (2). It is of interest to note that despite the tremendous difference in susceptibility to the lethal effect of CC between mice (LD_{50} 2.1 mg/kg; Fig. 7) and hamsters (LD_{50} 300 mg/kg; Ref. 22) the optimal dose of CC for the enhancement of antibody formation lies within the same range of about 1.0–1.5 mg/kg.

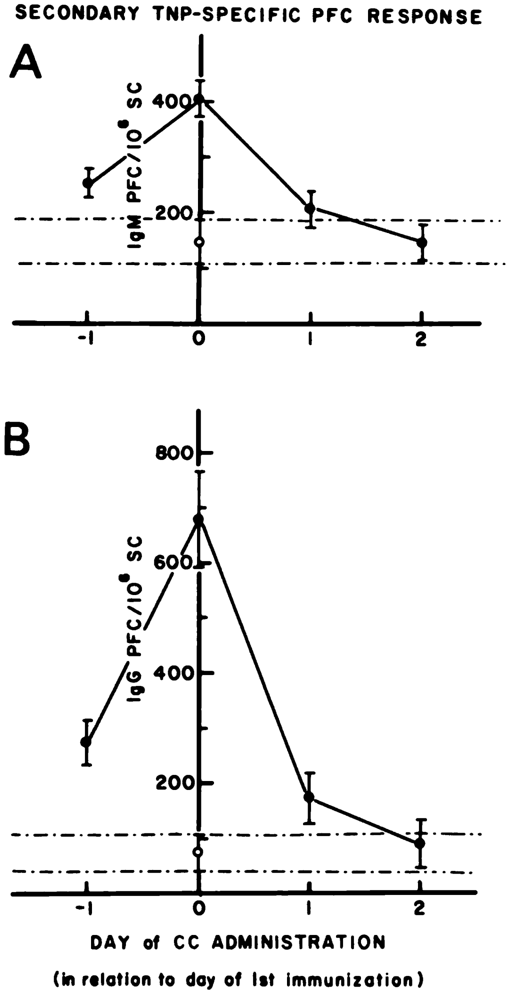

FIG. 10. Effect of varying the time of CC administration on the enhancement of the PFC response. Groups of mice (five animals per group) were given CC, at a dose of 1 mg/kg, on various days (as indicated on the abscissa) before, during, and after the immunization with 100 μg of TNP-KLH on day 0. Control animals received the same dose of antigen but no CC was given. 14 days after the first injection, all animals were challenged with 100 μg of TNP-KLH. The spleens of the injected animals were harvested and assayed for their TNP-specific PFC response 3 days after the second injection. Each point represents the mean response ± SE of the CC-treated animals (●) or of the control animals (○). SC, spleen cells.

The timing of the injection of CC seems to be critical. For the maximal enhancing effect, the drug has to be administered simultaneously with or on the same day as the antigen (Fig. 10). Only minimal enhancement of the PFC response was observed when CC was given 1 day before the injection of antigen. Effective enhancement had been reported in the guinea pig when CC was given as early as 6 h before antigen injection (9). On the other hand, administration of the drug 1 or 2 days after immunization was ineffective (Fig. 10). CC was also found to be incapable of enhancing the antibody response in rabbits when it was given 2 days before or 2 days after the injection of antigen (2). The failure of CC

TABLE I

Enhancement of the Primary Hapten-Specific PFC Response by CC and VB

Group	Number of mice	Day 0	Day 6			
			TNP-specific IgM PFC			
		Treatment	Per 10^6 SC	P*	Per spleen	P
			Mean ± SE			
I	12	TKB‡	125 ± 30		19,500 ± 3,010	
II	11	TKB + CC§	263 ± 74	<0.001	39,420 ± 6,630	<0.002
III	12	TKB + VB‖	248 ± 57	<0.002	38,290 ± 5,850	<0.002

* P values in comparison to group I.

‡ 100 μg TKB injected i.p.

§ CC administered at 1 mg/kg body weight, i.p.

‖ VB sulfate administered at 1 mg/kg body weight, i.p.

SC, spleen cells.

TABLE II

Effect of CC and LCC on the Primary Hapten-Specific PFC Response

Group	Number of mice	Day 0	Day 6			
			TNP-specific IgM PFC			
		Treatment	Per 10^6 SC	P*	Per spleen	P
			Mean ± SE			
I	6	TKB‡	170 ± 28		28,700 ± 5,090	
II	6	TKB + CC§	370 ± 32	<0.002	64,820 ± 6,950	<0.003
III	6	TKB + LCC‖	160 ± 41	n.s.	29,640 ± 7,910	n.s.

* P values in comparison to group I.

‡ 100 μg TKB injected i.p.

§ CC administered at 1 mg/kg body weight, i.p.

‖ LCC administered at 1 mg/kg body weight, i.p.

n.s., not significant; SC, spleen cells.

to enhance the antibody response when it was administered as early as 1 day after immunization suggests that certain critical regulatory cellular events must have taken place within 24 h after contact with antigen. Indeed, Eardley and Sercarz (23, 24) recently demonstrated in their elegant studies that regulatory suppressor cells appeared as early as 24 h after priming with antigen, and that cell divisions were required for the expression of suppressive activities. Taken together, these observations strongly support our hypothesis that CC may act on the early dividing suppressor cell or its precursors.

The route of CC administration does not seem to be important. In this study, we injected CC and the antigen separately into the peritoneal cavity. However, effective enhancement had also been observed when CC and the antigen were injected via different routes. For example, CC had been administered subcutaneously to rabbits immunized in the foot-pad (2), and intraperitoneally to hamsters immunized intravenously (21, 22); in both cases, antibody formation was increased.

If the enhancement of the antibody response by CC is dependent on the anti-mitotic action of the drug, then any anti-mitotic drug, e.g. VB, should also be capable of mediating the enhancement. Indeed, when VB was given to animals at the time of antigen injection, it was found to be as effective as CC in elevating the antibody response (Table I).

These observations with both CC and VB strongly suggest that there is a connection between the anti-mitotic action of the drug and its enhancement of antibody formation. The dose of CC (1-1.5 mg/kg) that we used to enhance the antibody response has been shown to be effective in inhibiting mitosis in vivo (25, 26). If the mitosis-blocking property is crucial for the enhancing effect of CC, deprivation of its anti-mitotic capacity should render it inactive in this regard. Indeed, we were able to show that the conversion of CC to its non-anti-mitotic structural isomer, LCC, abolished the enhancing effect (Table II). Thus, it seems justifiable to say that the anti-mitotic action of CC is an essential element for successful enhancement of the antibody response. The critical timing in the administration of CC necessary for effective enhancement, a feature of the effect of X-irradiation, indicates that these agents are most likely acting upon rapidly dividing cells. It is noteworthy that X-irradiation which also predominantly causes damage to dividing cells (27) also enhances the antibody response in vivo (28).

In contrast to the enhancing effect reported here, the mitosis-blocking capacity of CC has been employed to suppress the antibody response in rats by the administration of the drug a few days after immunization, but within several hours before the treated animals were sacrificed for the PFC assay (26). The effective suppressing dose (1.3 mg/kg) of CC used lies within our enhancing dose range (1.0-1.5 mg/kg). Thus, approximately the same dose of CC administered to animals may mediate either enhancement or suppression of antibody formation depending upon the timing of drug administration in relation to the injection of antigen. The suppressive effect of CC, observed when the drug is injected in the later phase of the antibody response, has been suggested to be the result of the mitotic blocking action of CC on cellular divisions of the antibody-forming cell and its precursors (26). On the other hand, we postulate that the enhancing effect of CC, observed when it is injected at the same time as antigen, is the consequence of its interruption of mitosis that prevents the generation of antigen-stimulated, early dividing suppressor cells.

It is clear, from the general effect of such anti-mitotic agents in rabbits, mice, guinea pigs, and hamsters on both the primary and secondary antibody responses to several protein antigens, that the early development of suppressor cells usually, if not always, accompanies antigenic stimulation. The accompanying paper presents evidence that CC does indeed prevent the development of suppressor cells.

Summary

Colchicine (CC) enhances the antibody response in mice to protein antigens, like diphtheria toxoid and human gamma globulin, as well as to the 2,4,6-trinitrophenyl hapten. Maximal enhancement was observed when CC was administered to animals on the same day as the injection of antigen. The

optimal dose of CC was in the range of 1.0–1.5 mg/kg body weight. The enhanced antibody formation was evident from elevated circulating antibody titers and from an increased number of antibody plaque-forming cells (PFC) of the spleen. The circulating antibody titer of CC-treated animals was higher than that of control animals by a factor of about 3–7 in the primary response, and by a factor of at least 15 in the secondary response. In terms of the number of antibody forming cells, CC enhanced the primary PFC response by ≅100%, and the secondary PFC response by as high as fivefold. The enhancing effect of CC seemed to be related to its mitosis-blocking capacity since (*a*) vinblastine, another antimitotic drug, was found to be as effective as CC and (*b*) lumicolchicine, the non-anti-mitotic structural isomer of CC, was ineffective in potentiating antibody responses. The critical timing in the administration of CC on the same day as antigen suggests that most likely, the mitotic poison was acting on antigen-stimulated early dividing suppressor cells.

The authors wish to thank Daniel Cargill for his excellent technical assistance.

Received for publication 28 October 1977.

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EFFECT OF COLCHICINE ON THE ANTIBODY RESPONSE

II. Demonstration of the Inactivation of Suppressor Cell Activities by Colchicine[*]

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Experiments reported in the preceding paper established the experimental conditions for the optimal enhancement of the antibody formation by colchicine (CC)[1] in mice (1). The effectiveness of CC in potentiating the antibody response raises the tantalizing question of how the drug acts to cause the enhancement. Since suppressor cells play an important role in the regulation of the antibody response (2–5), and the generation of these predominantly radiation-sensitive regulatory cells apparently requires mitosis (4, 6, 7), it is reasonable to assume that any interference with their proliferation or that of their precursors would reduce negative regulation. Considering the dose range (1.0–1.5 mg/kg) of CC which we find to be effective in enhancing antibody formation (1), one which has been shown to be anti-mitotic (8, 9), it is likely that CC exerts its mitosis-blocking effect on early dividing suppressor cells soon after their encounter with antigen (3, 7, 10, 11). Suppressor cells are thymus-derived (T) lymphocytes which suppress the antibody response in a number of systems (12–15). Thus it seems likely that in a system where T-cell regulation is absent, CC should be ineffective in enhancing the antibody response.

The random synthetic copolymer of L-glutamic acid^{50}-L-tyrosine^{50} (GT), which is not immunogenic in any of more than 20 inbred strains of mice, stimulates antibody responses when coupled to the immunogenic carrier, methylated bovine serum albumin (GT-MBSA) (16). Preimmunization with GT suppresses the plaque-forming cell (PFC) response to GT-MBSA in BALB/c mice. The specific suppressor activity has been shown to be mediated by T cells (17) and to be sensitive to cyclophosphamide (18). Therefore, in a system where specific suppressor T cells can be demonstrated, CC should be capable of preventing their activity.

Results obtained in the present study demonstrate that in the antibody response to a T-independent antigen, e.g. 2,4,6-trinitrophenyl–keyhole limpet hemocyanin (TNP-KLH)-Sepharose (19), CC effectively induces an elevated hapten-specific PFC response in intact animals, but not in congenitally athymic nude mice where T-cell regulation is absent. Furthermore, the generation of

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[1] *Abbreviations used in this paper:* CC, colchicine; GAT, L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}; GT, L-glutamic acid^{50}-L-tyrosine^{50}; KLH, keyhole limpet hemocyanin; MBSA, methylated bovine serum albumin; PFC, plaque-forming cells; SRBC, sheep erythrocytes; TKS, TNP-KLH-Sepharose; TNP, 2,4,6-trinitrophenyl.

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GT-induced suppressor cells and the transfer of their suppressive activity to normal syngeneic recipients can be effectively prevented by the administration of CC.

Materials and Methods

Animals. BALB/c mice were purchased from the same suppliers and maintained under the conditions described in the preceding paper (1).

Antigens and Immunization. The TNP-KLH used in this study was the same as that described previously (1). The hapten-carrier was conjugated to Sepharose 2-B (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) using the method described by Feldmann et al. (19). The synthetic random copolymer, GT, with an average mol wt of 133,000, as well as the terpolymer of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT), mol wt 35,000, were obtained from Miles Laboratories Inc., Miles Research Products, Elkhart, Ind. MBSA was purchased from Worthington Biochemical Corp., Freehold, N. J. The GT-MBSA complex was prepared by the dropwise addition of MBSA (5 mg/ml) while stirring, to a solution of GT (1 mg/ml). After a 20-min interval during which maximum flocculation and aggregation occurred, the precipitate of GT-MBSA was washed three times in saline. For immunization, mice were injected intraperitoneally with 10 μg of GT as GT-MBSA in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). CC (Sigma Chemical Co., St. Louis, MO.) was freshly dissolved in physiological saline and administered intraperitoneally to animals at a dose of 1 mg/kg body weight.

Cell Transfers. BALB/c mice used as cell donors were injected intraperitoneally with Maalox, 100 μg GT in Maalox, or 100 μg GT in Maalox and 1.0 mg CC/kg. 3 days later, mice were sacrificed and their spleens were removed. Single cell suspensions were prepared, washed three times in Hanks' balanced salt solution, and 20 × 10^6 lymphoid cells were adoptively transferred intravenously into normal syngeneic recipients. Mice were then immunized with GT-MBSA immediately after transfer.

Hemolytic Plaque Assay. The TNP-specific PFC response of immune animals was assessed as described previously (1). The PFC response to GT-MBSA was assayed on sheep erythrocytes (SRBC) coupled with the cross-reacting polymer GAT, GAT-SRBC (20). GT-specific plaques were determined by subtracting the number of PFC remaining after inhibition by a suitable dilution of GAT from the number of plaques detected on GAT-SRBC in the absence of the specific inhibitor (21).

Results

CC Enhances the PFC Response of Normal and Nude Mice to TNP-KLH-Sepharose. Although TNP-KLH is a thymus-dependent antigen, Feldmann and his colleagues have demonstrated that the conjugation of TNP-KLH to Sepharose (TKS) renders it thymus-independent (19). We performed experiments to test the effectiveness of CC in enhancing the antibody response to TKS in intact animals and in nude mice where T-cell regulation is absent. It can be seen from Fig. 1 that the simultaneous injection of CC with TKS into normal mice enhanced their hapten-specific PFC response. On the other hand, the administration of CC to congenitally athymic nude mice failed to increase their response (Table I).

CC Abrogates GT-Induced Suppression of the PFC Response to GT-MBSA. It has been established that preinjection of the copolymer GT suppresses the GT-specific PFC response of certain inbred mouse strains, e.g. BALB/c mice, to a challenge of the immunogenic GT-MBSA antigen (21). GT-induced suppression results from the stimulation of specific suppressor T cells (5, 17). We adopted this system to test the effect of CC on the generation of GT-stimulated suppressor cells. First, we examined the effectiveness of CC in blocking GT from inducing nonresponsiveness to GT-MBSA in BALB/c animals. It can be

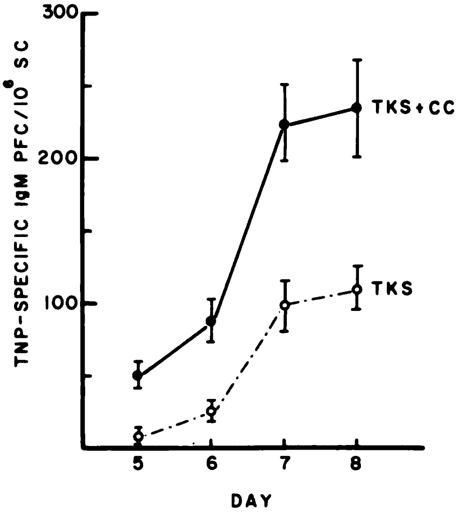

FIG. 1. Kinetics of the TNP-specific primary IgM PFC response of BALB/c mice to TKS. Animals in the control group (○) were immunized intraperitoneally with 100 μg of TKS on day 0, and animals in the experimental group (●) were similarly immunized, except that CC was also administered at a dose of 1 mg/kg body weight. On each day from day 5 to day 8 after the injection, spleens of the injected animals were assayed for their TNP-specific IgM PFC response. Each point represents the mean PFC ± SE of 5–10 animals. SC, spleen cells.

TABLE I

Effect of CC on the Primary Hapten-Specific PFC Response to TKS in BALB/c and Nude Mice

Group	Strain	Number of mice	Day 0	Day 7	P
			Treatment	TNP-specific IgM PFC per spleen	
				Mean ± SE	
I	BALB/c	10	TKS*	10,170 ± 1,310	
II	BALB/c	10	TKS + CC‡	25,980 ± 3,190	<0.003§
III	Nude (nu/nu)	6	TKS	8,500 ± 1,200	
IV	Nude (nu/nu)	6	TKS + CC	9,290 ± 1,020	n.s.‖

* 100 μg of TKS injected i.p.

‡ CC administered at 1 mg/kg body weight, i.p.

§ P value in comparison to group I.

‖ n.s., not significant in comparison to group III.

seen from Table II that prior injection of GT effectively suppressed the GT-specific PFC responses of the injected animals in group II to a subsequent challenge with GT-MBSA. On the other hand, animals in group III, preinjected with GT as well as CC, developed a PFC response to GT-MBSA no different from that of control animals in group I.

Results of experiments described above strongly imply that CC may abolish the GT-induced suppression by inactivating the suppressor T cells. If this

TABLE II

Effect of CC on GT-Induced Suppression of the PFC Response to GT-MBSA in BALB/c Mice

Group	Number of mice	Day −3	Day 0	Day 7	*P**
		Treatment		GT-specific PFC per spleen	
				Mean ± SE	
I	9	Maalox	GT-MBSA‡	4,240 ± 770	
II	9	GT§	GT-MBSA	180 ± 20	<0.001
III	8	GT + CC‖	GT-MBSA	4,180 ± 400	n.s.¶

* *P* values in comparison to group I.

‡ 10 μg of GT as GT-MBSA in complete Freund's adjuvant, i.p.

§ 100 μg of GT in Maalox, i.p.

‖ CC administered at 1 mg/kg body weight, i.p.

¶ n.s., not significant.

TABLE III

Effect of CC on the Transfer of Suppression of GT-Specific PFC Response to Normal Syngeneic Recipients (BALB/c Mice)

Group	Day −3	Day 0		Day 7	Number of mice	*P**
	Donors	Normal sygeneic recipients		GT-specific IgG PFC per spleen		
	Treatment	Spleen cells transferred	Immunization			
				Mean ± SE		
I	Maalox	20×10^6	GT-MBSA‡	5,780 ± 930	12	
II	GT§	20×10^6	GT-MBSA	500 ± 160	12	<0.001
III	GT + CC‖	20×10^6	GT-MBSA	6,150 ± 1,040	12	n.s.¶

* *P* values in comparison to group I.

‡ 10 μg of GT as GT-MBSA in complete Freund's adjuvant, i.p.

§ 100 μg of GT in Maalox, i.p.

‖ CC administered at 1 mg/kg body weight, i.p.

¶ n.s., not significant.

implication were correct, one would predict that the administration of CC to GT-primed donors should prevent the transfer of suppressor activity to normal syngeneic recipients. It can be seen from Table III that the transfer of GT-primed spleen cells to normal syngeneic recipients immunized with GT-MBSA significantly suppressed the IgG PFC response to GT-MBSA (compare groups II and I). In contrast, the injection of CC to cell donors at the time of priming with GT abolished the transfer of suppressor activity to normal recipients challenged with GT-MBSA (compare groups III and II). These results suggested that CC abolished GT-specific suppression by the inactivation or elimination of GT-stimulated suppressor cells.

Discussion

Studies reported in the preceding paper clearly established the effect of CC in enhancing the antibody response in mice (1). Results of experiments presented in this paper have demonstrated that (*a*) CC is ineffective in potentiating antibody formation in mice in which T-cell regulation is absent and (*b*) CC abolishes the expression of GT-induced suppressor cell activities.

It could be argued that CC induces an elevated antibody response by its direct effect on B cells. Hence, we tested its action on congenitally athymic mice to a T-independent antigen, TKS. Our results indicate that CC did not enhance antibody formation in homozygous nude (nu/nu) mice where T-cell regulation is absent (Table I). In contrast, the hapten-specific PFC response of intact animals to TKS can be enhanced by CC (Fig. 1). Although it has been shown by Feldmann et al. (19) that helper T cells and macrophages are not required to elicit a hapten-specific response to TKS, the magnitude of the response might still be under the control of regulatory T cells, e.g. suppressor cells. The effectiveness of CC in elevating the antibody response to TKS in intact animals where T-cell regulation is present, and the failure of the drug to enhance the response to the same T-independent antigen in T-cell-deficient mice strongly imply that under our experimental conditions, one of the target cells acted upon by CC is a regulatory T cell. Our observation is analogous to the report by Baker et al. (22, 23) that anti-lymphocyte serum enhanced the response to type III pneumococcal polysaccharide, a T-independent antigen, in intact animals but not in T-cell-deficient nude animals.

Although results up to this point suggested that CC acts on suppressor cells, direct evidence to support the view was lacking. To demonstrate that CC in fact inactivates suppressor cells, we used a well-established system in which specific suppressor T cells are induced by the injection of GT in BALB/c animals (5, 21). It is clear from these experiments that the simultaneous injection of CC with GT effectively prevented the induction of GT-specific suppression of PFC response to GT-MBSA (Table II). Furthermore, the administration of CC to GT-primed donor animals abolished the cell-mediated transfer of suppressive activity to normal syngeneic recipients challenged with GT-MBSA (Table III). This observation provided direct evidence that CC inactivated or eliminated suppressor T cells. It is noteworthy that GT-specific suppression in BALB/c mice can also be abolished by pretreatment with cyclophosphamide (18).

The selective action of CC on the inactivation of suppressor cells or their precursors appears to be a matter of the appropriate time of its administration. Recent studies by Eardley and Sercarz (10) demonstrated that a wave of suppression occurs as the initial event immediately following immunization in a hapten-carrier system, and that carrier-specific suppressor cells are generated as early as 24 h after the injection of antigen. Moreover, such suppression required cell division (7). Hence, the finding that simultaneous administration of CC with an antigen consistently enhances the antibody response (1), and that CC is capable of inactivating suppressor cell activities (Tables II and III), strongly substantiate the view that CC interferes with the generation of the initial wave of suppression, and this in turn leads to a subsequent augmentation of the antibody response.

Of course, the demonstration that CC acts on suppressor cells does not exclude the possibility that it may also be acting on other cell types. For example, we cannot rule out as yet its possible effect on macrophages, and its possible action on helper T cells in the enhancing process. A possible contribution by macrophages in enhancing the antibody response may be partly the result of increased efficiency of macrophages in processing the antigen. However, CC has been shown to inhibit phagocytosis (24–26) and to interfere with certain metabolic processes in particle ingestion (24, 27). Thus, these observations on the effect of CC upon neutrophil and macrophage functions suggest that the administration of CC with an antigen probably would not significantly facilitate macrophages in antigen processing. It also appears unlikely to us that any interference with the generation of helper T cells by an anti-mitotic dose of CC would facilitate rather than impair helper function. In view of our current finding that the early administration of CC in relation to antigen injection is a prerequisite for successful enhancement of the antibody response, we tend to favor the alternative possibility that suppressor cells or their precursors, which appear early after antigenic stimulation, are its principal targets.

Summary

The simultaneous administration of colchicine (CC) with a T-independent antigen, e.g. 2,4,6-trinitrophenyl–keyhole limpet hemocyanin-Sepharose, to intact animals effectively enhanced their hapten-specific plaque-forming cell (PFC) response. However, in congenitally athymic nude mice in which T-cell regulation was absent, CC was ineffective in producing enhancement. These observations suggest that the target cell acted upon by CC is most likely thymus-derived. Furthermore, the injection of CC with the co-polymer of L-glutamic acid^{50}-L-tyrosine^{50} (GT) abolished GT-specific suppression of the PFC response to GT-methylated bovine serum albumin. Spleen cells from CC-treated and GT-primed hosts could no longer transfer suppressive activity to normal recipients. These results provide evidence that CC is capable of inactivating or eliminating suppressor cells or their precursors. Thus, CC-induced enhancement of the antibody response may be explained, at least in part, by its anti-mitotic, and hence lethal effect on dividing suppressor T cells.

The authors wish to thank Daniel Cargill and Linda Kapp for their excellent technical assistance.

Received for publication 28 October 1977.

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ROLE OF VIRAL INFECTIVITY IN THE INDUCTION OF INFLUENZA VIRUS-SPECIFIC CYTOTOXIC T CELLS

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Cellular immune responses have been shown to play an important role in determining the outcome of virus infection in several experimental models (1, 2). Among the manifestations of cellular immunity in viral infection the capacity of specifically sensitized thymus-derived (T)[1] lymphocytes to destroy virus-infected target cells in vitro has been demonstrated to correlate with elimination of infectious virus in vivo (3, 4) and hence suggests a direct role for cytotoxic T lymphocytes (CTL) in recovery from virus infection. Since the functional activity of CTL raised against viruses (5–8) as well as minor histocompatibility antigens (9, 10) and chemically modified cells (11) has also been shown to be under the control of genes in the major histocompatibility complex (MHC), the specificity of CTL for both the gene products of the MHC and the foreign determinant on target cell surfaces has been analyzed in great detail. On the other hand, much less information is presently available on the requirements for induction of virus-specific CTL. An issue which is presently controversial is the requirement for infectious virus in the induction of virus-specific CTL. Several laboratories have reported that CTL responses to a diverse group of viruses can be readily stimulated with inactivated virion preparations (12–15), whereas infectious virus is required for CTL induction in other hands (16). Also inactivated virus (12, 13) or indeed virion subunit preparations (17) have been reported to sensitize target cells for CTL-mediated lysis in the absence of nascent viral protein in the target cell (12, 17), whereas infectious virus and nascent viral protein synthesis is required for lysis of target cells in other circumstances (18). The resolution of this issue would appear to have direct implications with respect to viral vaccines, particularly in assessing the efficacy of live virus and killed virus vaccines. Furthermore, an understanding of the role of viral infectivity both in the induction of CTL and target cell sensitization may be of value in understanding the mechanism of CTL recognition.

This report examines the capacity of infectious and inactivated influenza virus to stimulate virus-specific CTL responses in vivo and in vitro. We have

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* Recipient of a postdoctoral fellowship from the Jane Coffin Childs Memorial Fund for Medical Research.

[1] *Abbreviations used in this paper:* CTL, cytotoxic thymus-derived lymphocyte(s); EID_{50}, egg infectivity dose yielding 50% positive response; HAU, hemagglutination units of virus; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; T cell, thymus-derived lymphocyte; UV, ultra-violet.

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observed that while infectious influenza virus was capable of stimulating both CTL responses and humoral immune responses in vivo over a broad immunizing dose range, ultra-violet inactivated influenza virus neither induced CTL responses in vivo nor sensitized putative target cells for lysis by influenza-specific CTL in spite of its capacity to stimulate a comparable in vivo humoral immune response. Inactivated influenza virus could, however, stimulate a CTL response in vitro. These results are discussed in the light of the observations outlined above. Possible implications for virus-specific CTL induction are also considered.

Materials and Methods

General. Male BALB/C mice (7-12 wk of age) bred at the John Curtin School were used throughout. P815 mastocytoma cells, maintained in tissue culture, were used as target cells in all experiments (19). Eagle's minimal essential medium (Grand Island Biological Co., Grand Island, N.Y. catalogue no. F-15), supplemented with 10% heat-inactivated fetal calf serum (Commonwealth Serum Laboratories, Melbourne, Australia) was used as the medium in all cytotoxicity assay.

Viruses. Influenza virus strains A/WSN (H0/N1), A/JAP/305 (H2/N2), and B/LEE were grown in the allantoic cavity of embryonated eggs and stored as infectious allantoic fluid as described previously (19).

Virus Purification and Inactivation. Before inactivation, virus, as infectious allantoic fluid, was concentrated and purified according to standard procedures (20). Influenza A/WSN was concentrated by adsorption-elution from fowl erythrocytes followed by ultracentrifugation. Influenza A/JAP was similarly concentrated and further purified by velocity centrifugation over sucrose gradients (20). Purified virus was diluted to a concentration of 10^5 hemagglutination units (HAU)/ml in sterile phosphate-buffered saline (PBS) and exposed to a 25-watt ultraviolet (UV) light source at a distance of 20 cm for 7 min in 9 cm glass Petri dishes containing 5-6 ml of virus suspension. The virus suspension was stirred constantly during exposure to the UV source. The infectious viruses had a titer of 5×10^8 -10^9 EID_{50} U/ml and 1.5-3.0×10^3 HAU/ml. Egg infectivity titers were determined by the modified Spearman-Käber method (21). Hemagglutination titrations were performed according to Fazekas de St. Groth and Webster (22). No residual infectious virus was detectable in inactivated virus preparations as measured by egg infectivity. No loss of viral hemagglutinating activity was observed after virus inactivation. Inactivated virus was stored at 4°C.

Immunization. Mice were inoculated with virus by the intravenous route. Infectious virus, as allantoic fluid was diluted in PBS and administered in quantities as indicated in the text. UV-inactivated virus was administered in a similar fashion. For in vivo primary cytotoxic responses, spleens from three donor mice were removed 6 days after immunization and a spleen cell suspension was prepared as previously described (8). For in vivo secondary responses, mice primed previously with 100 HAU of infectious virus were inoculated 3-4 wk later with infectious or inactivated virus as indicated in the text. 5 days later spleens from three donors were removed and processed as above. For adoptive in vivo secondary responses 80-100 × 10^6 spleen cells from donors primed 3-4 wk previously with infectious virus were transferred intravenously into age and sex matched recipient mice which had received 450 rads of total body γ-irradiation from a radioactive cobalt source. Recipient mice were inoculated with virus immediately after cell transfer. Recipient spleens were removed 5 days later and tested for cytotoxicity.

In Vitro Secondary Responses. Cytotoxic T lymphocytes were generated in vitro essentially as described previously (8, 19). Briefly, 40 × 10^6 spleen cells from mice primed 3-8 wk previously with 100 HAU of infectious influenza virus were cultured with "stimulator" cells in 25 cm^2 Falcon tissue culture flasks (Becton, Dickinson and Co., Oxnard, Calif.) containing 15 ml of medium (8, 19). The responder cell to stimulator cell ratio was 10:1. Stimulator cells consisted of normal syngeneic spleen cells either infected with 5 EID_{50} U of infectious virus per nucleated cell (60 HAU of infectious virus per 4 × 10^6 cells) or treated with various quantities of inactivated influenza virus in an identical fashion. Stimulator cells were washed twice before culturing with responder cells to remove unadsorbed virus. Viable cells were tested for cytotoxic activity after 5 days of culture. Recovery of viable cells was 20-40% at that time.

Assay for Cell-Mediated Cytotoxicity. The ^{51}Cr release cytotoxicity assay was carried out as described in previous reports (8, 19). ^{51}Cr-labeled P815 target cells were infected in suspension with 10 EID_{50} U of infectious virus per cell (30 HAU of infectious virus per 10^6 target cells) as described (19). Target cells were treated with various quantities of UV-inactivated influenza virus in an identical fashion. Unless otherwise indicated in the text, assay times were 8-8.5 h for primary effector activity and 6.5 h for secondary effectors. Spontaneous ^{51}Cr release from target cells incubated with medium only usually ranged from 5 to 15% and was always less than 18%. Percent specific ^{51}Cr release was obtained from the formula:

$$\frac{\text{test counts} - \text{spontaneous release}}{\text{water lysis counts} - \text{spontaneous release}} \times 100.$$

All values represent the mean percent specific ^{51}Cr release of four replicate wells.

Assay for Anti-Hemagglutinin Antibody. Serum anti-hemagglutinin antibody was quantitated by the microtitration hemagglutination inhibition test (23). 4 HAU of virus in a vol 0.025 ml were added to serial twofold dilutions of serum in a final vol of 0.025 ml of PBS. After 35 min of incubation, 0.025 ml of a 1% suspension of fowl erythrocytes was added to each well. After 30 min of incubation, the hemagglutination-inhibition endpoint was determined. All sera were treated with *Vibrio cholera* receptor-destroying enzyme (Center for Disease Control, Atlanta, Ga.) and heated to 56°C for 30 min to remove nonspecific inhibitors. Preimmune sera from immune sera donors served as controls.

Results

In a series of preliminary experiments the capacity of influenza virus strain A/WSN to induce CTL was assessed after inactivation of the virus by several different methods. In contrast to results obtained with infectious influenza virus (8, 19) no influenza-specific CTL activity was detectable in the spleens of mice after intravenous inoculation of A/WSN virus inactivated either by UV irradiation, sodium deoxycholate disruption, or heat treatment (data not shown). Because UV irradiation was considered to have the least detrimental effect on both virion architecture and viral antigen stability, this method of virus inactivation was used in subsequent experiments.

Antigen Dose Dependence of the in Vivo Primary CTL Response to Infectious Influenza Virus. Fig. 1 shows the cytotoxic response from the spleens of mice 6 days after administration of the indicated doses of infectious influenza A/WSN. Cytotoxic activity was detectable with infectious virus doses as low as 10^{-3} HAU (10^2 EID_{50} U). The magnitude of lytic activity was directly proportional to the concentration of infectious virus in the immunizing inoculum over a range of antigen doses. This direct relationship between immunizing virus dose and splenic CTL activity was consistently observed in a series of experiments. An analysis of the kinetics of the cytotoxic response (not shown) indicated that as demonstrated previously (8, 19, 24) optimal cytotoxic activity was maximum at 5-7 days postinoculation. Thus, the difference in magnitude of the cytotoxic response with different virus doses was not attributable to differences in the kinetics of appearance of cytotoxic activity. The T-cell origin of the cytotoxic cell activity has been demonstrated previously (8, 19).

Absence of an in Vivo Primary CTL Response with UV-Inactivated Influenza Virus. Table I shows a comparison CTL response of mice 6 days after i.v. inoculation with various concentrations of infectious or UV-inactivated influenza A/WSN. In contrast to infectious virus, UV-inactivated A/WSN virus did

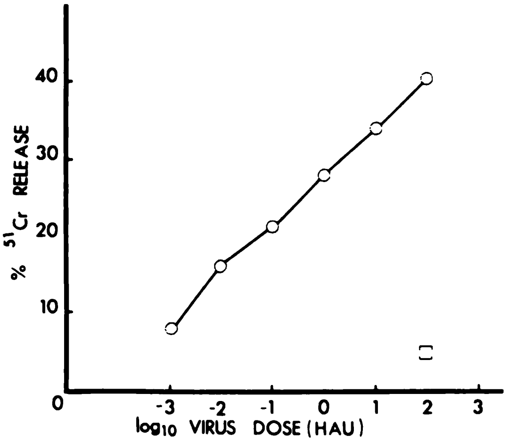

FIG. 1. Antigen dose dependence of the in vivo primary cytotoxic T-cell response to infectious influenza virus A/WSN. Spleen cells from pools of three mice were obtained 6 days after primary immunization with the indicated virus dose (abscissa) and tested for cytotoxicity on ^{51}Cr-labeled A/WSN infected target cells (O——O). The cytotoxic activity of normal spleen cells is also included (□). The effector cell: target cell ratio is 50:1. Values are the mean of four individual wells. Standard errors less than ± 3% in all cases have been omitted.

not generate a significant CTL response in vivo with virus doses as high as 10^4 HAU. The low level of cytotoxicity observed in the assay at the highest effector to target ratio is comparable to the background cytotoxicity observable with normal spleen cells and probably does not reflect low level specific cytotoxic activity. Spleens from mice immunized with 10^3 HAU of inactivated virus were also examined for CTL activity at 2-day intervals up to 10 days after immunization. No cytotoxic activity was detectable during this period at a time when optimal CTL responses are detectable in a variety of diverse viral systems (25). Although these results were obtained with influenza strain A/WSN, we have obtained similar results with two other influenza strains: A/JAP/305 (H2N2) and B/LEE.

Humoral Immune Response to Infectious and Inactivated Influenza Virus. Since the above results indicated a marked disparity between infectious and UV-inactivated virus in their respective capacities to induce CTL responses in vivo, the humoral immune response to various doses of these virus prepartions was examined (Table II). Both virus preparations induced significant levels of anti-viral antibody as measured by hemagglutination inhibition. Likewise for both virus preparations the magnitude of the response was proportional to the immunizing antigen dose. Although the humoral response to infectious virus was greater at lower immunizing doses, similar antibody levels were achieved upon immunization with higher doses of UV-inactivated virus, i.e., 10^2-10^4 HAU.

Absence of an in Vivo Secondary CTL Response on Challenge with Inactivated Influenza Virus. Secondary CTL responses to infectious influenza virus have been demonstrated both in vivo (24) and in vitro (8, 19) after primary immunization with infectious influenza virus. Since the above results indicated that UV-inactivated influenza was a poor stimulator of a primary CTL response,

TABLE I

Comparison of the in Vivo Primary Cell-Mediated Cytotoxic Response to Infectious and Inactivated Influenza Virus[*]

Virus dose[‡]	Effector cell to target cell ratio[§]	% Specific ^{51}Cr release on A/WSN infected P815 target cells[¶] Immunization[‖]	
		Infectious virus	Inactivated virus
10^{-2}	25:1	19.5 ± 0.4[**]	NT[‡‡]
	50:1	43.4 ± 1.9	"
	100:1	48.4 ± 2.7	"
10^{-1}	25:1	43.3 ± 1.5	0.1
	50:1	59.5 ± 0.5	1.0 ± 0.1
	100:1	57.5 ± 2.9	3.1 ± 0.1
10^{0}	25:1	47.0 ± 1.8	0.6
	50:1	62.5 ± 1.2	2.0 ± 0.1
	100:1	74.6 ± 0.6	3.8 ± 0.1
10^{1}	25:1	60.1 ± 1.2	0.7
	50:1	69.5 ± 2.5	2.7 ± 0.1
	100:1	79.3 ± 4.6	6.3 ± 0.2
10^{2}	25:1	64.0 ± 1.1	1.7 ± 0.1
	50:1	73.8 ± 1.2	3.6 ± 0.3
	100:1	81.7 ± 1.9	7.3 ± 0.3
10^{3}	25:1	NT	1.5 ± 0.1
	50:1	"	2.9 ± 0.1
	100:1	"	5.2 ± 0.3
10^{4}	25:1	NT	3.0 ± 0.1
	50:1	"	6.0 ± 0.2
	100:1	"	8.5 ± 0.2

[*] BALB/c mice were immunized i.v. with the indicated dose of infectious or UV-inactivated influenza A/WSN. 6 days later spleen cells from pools of three mice were assayed for cytotoxicity.

[‡] Virus dose employed in primary immunization measured in HAU. 1 HAU of infectious virus contains 1.7–3.0 × 10^5 EID_{50} U of infectious virus.

[§] 2 × 10^4 ^{51}Cr-labeled P815 cells/well.

[¶] All effector populations were simultaneously examined on target cells infected with A/WSN and B/LEE viruses. ^{51}Cr release values on B/LEE infected targets were <10% and <2% for cells obtained from donors receiving infectious and inactivated virus respectively.

[‖] Indicates source of spleen cells, i.e. from donors receiving infectious or inactivated virus.

[**] Means ± standard errors of the mean from four wells with spontaneous release subtracted.

[‡‡] Not tested.

TABLE II
*Primary Serum Anti-Hemagglutinin Antibody Response to Infectious and Inactivated Influenza Virus**

Virus dose‡	Anti-hemagglutinin antibody titer§ Immunization‖	
	Infectious virus	Inactivated virus
10^4	NT	160 ± 0¶
10^3	160 ± 0	NT
10^2	160 ± 0	80 ± 0
10^1	113 ± 11	NT
10^0	95 ± 10	16 ± 11
10^{-1}	73 ± 11	NT
10^{-2}	20 ± 11	<10

* Groups of four mice were immunized i.v. with the indicated dose of infectious or UV-inactivated influenza A/WSN. 10 days later, individual mice were bled from the tail vein and the serum hemagglutination inhibiting antibody titer determined.

‡ As in Table I.

§ Hemagglutination inhibiting antibody titer as measured in the microtitration hemagglutination-inhibition test.

‖ Indicates source of serum, i.e. from mice receiving infectious or inactivated virus.

¶ Values are the reciprocals of means ± standard errors of the highest serum dilution giving complete inhibition of hemagglutination from four individual mice.

it was of interest to examine the capacity of inactivated virus to induce a CTL response in mice previously primed with infectious influenza virus. Mice immunized 3–4 wk previously with 100 HAU of infectious influenza A/WSN or A/JAP were challenged with 1,000 HAU of UV-inactivated A/WSN or A/JAP virus. Control mice received no further treatment beyond primary immunization. After 5 days the cytotoxic activity of spleen cells from the various groups was examined on target cells infected with A/WSN, A/JAP, B/LEE, or uninfected target cells (Table III). In no instance did the cytotoxic activity of spleen cells from mice receiving secondary challenge with inactivated influenza virus exceed the background cytotoxicity of cells from control mice. On the other hand, spleen cells from mice previously primed with infectious A/WSN virus showed significant cytotoxic activity on both A/WSN and A/JAP infected target cells when secondarily stimulated with infectious A/JAP virus (Table III).

Generation of an in Vitro Secondary Response to Inactivated Influenza Virus. Although the above results would indicate that inactivated influenza is also an inefficient stimulator of secondary CTL responses, the presence of circulating anti-viral antibody in the primed recipients could alter the secondary CTL response upon challenge with inactivated virus. Indeed, it has been demonstrated that poor secondary CTL responses are observed when mice, previously primed with a given infectious type A influenza strain are challenged with the homologous infectious virus (24), whereas secondary stimulation with an infectious type A influenza strain of a different subtype generates good CTL responses in the primed recipients (Table III and [24]). This poor cytotoxic T-cell response on secondary stimulation with homologous virus appears to be due to

TABLE III

In Vivo Secondary Cell-Mediated Cytotoxic Response to Inactivated Influenza Virus

Immunization[*]		% Specific ^{51}Cr release from target cells[‡]			
Primary	Secondary	Uninfected	A/WSN	A/JAP	B/LEE
A/WSN	A/WSN (Inactivated)	2.5 ± 0.1	13.5 ± 0.7	9.3 ± 0.3	0
A/WSN	A/JAP (Inactivated)	2.4 ± 0.1	18.4 ± 0.9	12.0 ± 1.0	2.5 ± 0.2
A/WSN	None	0	17.3 ± 0.8	14.7 ± 0.4	0.7
A/JAP	A/WSN (Inactivated)	3.9 ± 0.2	9.8 ± 0.3	11.5 ± 0.5	0.8
A/JAP	A/JAP (Inactivated)	0	7.6 ± 0.1	13.3 ± 0.3	0.3
A/JAP	None	0	12.5 ± 0.6	10.3 ± 0.4	0.7
A/WSN	A/JAP (Infectious)	0	60.4 ± 1.4	54.3 ± 1.5	0.3

[*] 3-4 wk after primary immunization with infectious A/WSN or A/JAP virus mice were challenged with either inactivated A/WSN or A/JAP virus as indicated in the text. Controls consisted of mice receiving no secondary immunization and A/WSN primed mice challenged with 100 HAU of infectious A/JAP virus. 5 days later, spleen cells from pools of three mice were assayed for cytotoxic activity on the indicated target cells. Effector cell:target cell ratios were 100:1.

[‡] Values are the means ± standard errors of the mean from four wells with spontaneous release subtracted.

neutralization of the infectious virus inoculum by circulating antibody in the primed recipient. Since highly potent secondary CTL responses to homologous type A influenza virus can be obtained by in vitro secondary stimulation of primed cells (8, 19), we examined the capacity of inactivated virus to stimulate a secondary CTL response in vitro where the problem of circulating anti-viral antibody could be circumvented. Spleen cells from mice previously immunized with infectious A/JAP virus were cultured with normal spleen cell stimulators which had been treated with either inactivated A/JAP virus, inactivated A/WSN virus, or infectious A/JAP, A/WSN, or B/LEE viruses. After 5 days the cytotoxic activity of the cultured cells was examined (Table IV). In contrast to the results obtained above, spleen cells from A/JAP-primed mice generated potent cytotoxic effector cells when stimulated in vitro with UV-inactivated A/JAP virus. Furthermore, the magnitude of the response was dependent upon the dose of stimulating antigen. Also no response was observed when normal spleen cells, treated with inactivated A/WSN virus, were used for in vitro stimulation. It is of interest to note the cytotoxic activity generated by A/JAP primed cells after stimulation with inactivated A/JAP was directed exclusively to A/JAP-infected target cells. There was no lysis of A/WSN infected targets above the background seen with uninfected target cells or target cells infected with the serologically unrelated B/LEE influenza virus. This observation is in contrast to the finding with infectious virus where, as has been previously shown (19), stimulation of A/JAP primed cells with stimulator cells treated (infected) with infectious A/JAP or A/WSN virus generates CTL which can efficiently lyse both A/WSN and A/JAP infected targets. Finally, there was no

TABLE IV

In Vitro Secondary Cell-Mediated Cytotoxic Response to Inactivated Influenza Virus

In vitro stimulation[*]	Effector cell to target cell ratio[§]	% Specific ^{51}Cr release from target cells[‡]			
		Uninfected	A/WSN	A/JAP	B/LEE
A/JAP-Inactivated (25 HAU)[‖]	1:1	0.4	1.3 ± 0.1	3.4 ± 0.3	0
	2.5:1	0.5	1.8 ± 0.1	9.8 ± 0.3	1.6 ± 0.1
	5:1	2.2 ± 0.1	3.3 ± 0.1	18.3 ± 0.5	4.3 ± 0.2
A/JAP-Inactivated (250 HAU)	1:1	0	1.1 ± 0.1	18.8 ± 0.1	1.1 ± 0.1
	2.5:1	1.4 ± 0.1	3.5 ± 0.2	43.0 ± 1.3	2.4 ± 0.1
	5:1	3.2 ± 0.1	5.8 ± 0.2	61.9 ± 1.3	4.8 ± 0.2
A/JAP-Inactivated (2,500 HAU)	1:1	2.0 ± 0.1	3.4 ± 0.1	41.5 ± 1.5	2.2 ± 0.1
	2.5:1	5.4 ± 0.2	8.8 ± 0.4	66.5 ± 1.7	6.9 ± 0.2
	5:1	10.5 ± 0.2	15.2 ± 0.3	79.7 ± 1.5	14.3 ± 0.4
A/WSN-Inactivated (2,500 HAU)	1:1	0	0	1.3 ± 0.1	1.0
	2.5:1	2.2 ± 0.1	2.6 ± 0.1	3.1 ± 0.1	2.3 ± 0.1
	5:1	4.1 ± 0.2	5.2 ± 0.1	4.3 ± 0.1	4.5 ± 0.2
A/JAP-Infectious	1:1	4.5 ± 0.2	42.6 ± 1.2	60.2 ± 1.5	4.8 ± 0.1
	2.5:1	10.3 ± 0.5	58.2 ± 1.2	73.2 ± 2.7	9.7 ± 0.3
	5:1	16.5 ± 0.4	66.2 ± 2.1	78.6 ± 2.4	18.7 ± 0.2
A/WSN-Infectious	1:1	1.7 ± 0.1	30.5 ± 1.1	34.3 ± 1.9	1.5 ± 0.1
	2.5:1	4.1 ± 0.3	55.4 ± 1.8	59.8 ± 1.3	3.4 ± 0.1
	5:1	8.0 ± 0.6	62.0 ± 1.0	80.2 ± 0.6	7.2 ± 0.2
B/LEE-Infectious	1:1	2.1 ± 0.1	3.2 ± 0.1	2.6 ± 0.1	2.3 ± 0.1
	2.5:1	4.6 ± 0.3	4.9 ± 0.1	4.5 ± 0.3	5.2 ± 0.1
	5:1	6.9 ± 0.3	7.3 ± 0.1	7.7 ± 0.3	7.3 ± 0.4
–[¶]	1:1	3.4 ± 0.2	2.0 ± 0.1	0.7	1.7 ± 0.1
	2.5:1	4.5 ± 0.3	2.3 ± 0.1	1.7 ± 0.1	2.1 ± 0.1
	5:1	4.2 ± 0.2	3.9 ± 0.1	3.6 ± 0.1	2.9 ± 0.2

[*] Spleen cells from A/JAP-immune mice were cultured in vitro with stimulator spleen cells treated with infectious or inactivated influenza virus as described (Materials and Methods). After 5 days of culture, the cytotoxic activity of the responder cells was examined.

[‡] As in Table III.

[§] As in Table I.

[‖] Parentheses indicate dose of inactivated virus incubated with stimulator spleen cells.

[¶] Indicates normal stimulator spleen cells not exposed to virus.

cytotoxic activity detectable in cultures stimulated with untreated or influenza B/LEE infected stimulators.

Absence of an in Vivo Secondary CTL Response to Inactivated Virus after Adoptive Transfer. Two points emerge from the results obtained with secondary stimulation in vitro: (*a*) inactivated virus is capable of stimulating a secondary CTL response in vitro; (*b*) the response is observed only when in vitro stimulation is carried out with the homologous virus strain used in primary immunization and the cytotoxic activity is directed exclusively to the target cells infected with the homologous virus strain. These in vitro observa-

TABLE V

Adoptive in Vivo Secondary Cytotoxic Response to Infectious and Inactivated Influenza Virus

Immune cells transferred*	Secondary stimulation‡	Effector cell to target cell ratio§	% Specific ^{51}Cr release from target cells‖			
			Uninfected	A/WSN	A/JAP	B/LEE
A/WSN	A/WSN-Inactivated	25:1	1.5 ± 0.1	5.0 ± 0.2	3.7 ± 0.1	4.6 ± 0.1
		50:1	1.3 ± 0.1	6.8 ± 0.2	4.4 ± 0.1	4.3 ± 0.1
A/WSN	A/JAP-Inactivated	25:1	1.0	2.5 ± 0.1	1.6 ± 0.1	3.9 ± 0.1
		50:1	2.0 ± 0.1	5.4 ± 0.1	3.9 ± 0.1	4.8 ± 0.3
A/WSN	A/WSN-Infectious	25:1	2.5 ± 0.1	69.4 ± 1.9	32.7 ± 1.9	4.5 ± 0.1
		50:1	3.9 ± 0.1	83.6 ± 3.1	50.4 ± 2.9	6.8 ± 0.2
A/WSN	None	25:1	1.7 ± 0.1	1.7 ± 0.1	2.0 ± 0.1	2.7 ± 0.1
		50:1	2.1 ± 0.1	4.7 ± 0.1	3.8 ± 0.1	4.2 ± 0.1
A/JAP	A/WSN-Inactivated	25:1	3.7 ± 0.1	3.5 ± 0.1	6.3 ± 0.1	6.1 ± 0.2
		50:1	4.0 ± 0.1	6.6 ± 0.4	8.1 ± 0.1	6.7 ± 0.2
A/JAP	A/JAP-Inactivated	25:1	4.4 ± 0.2	5.8 ± 0.2	8.1 ± 0.2	6.5 ± 0.5
		50:1	5.9 ± 0.1	6.7 ± 0.3	10.5 ± 0.5	7.1 ± 0.3
A/JAP	A/JAP-Infectious	25:1	6.1 ± 0.3	45.7 ± 2.0	47.2 ± 0.5	8.5 ± 0.6
		50:1	8.4 ± 0.3	63.2 ± 2.6	63.5 ± 1.7	12.2 ± 0.6
A/JAP	None	25:1	6.2 ± 0.2	5.5 ± 0.2	8.1 ± 0.1	8.0 ± 0.4
		50:1	8.5 ± 0.1	10.8 ± 0.2	12.2 ± 0.1	10.3 ± 0.1

* Spleen cells from mice previously primed with the indicated infectious virus were transferred into sublethally irradiated (450 rads) mice. Recipient mice were immediately challenged with the indicated virus as described (Materials and Methods). Recipient spleens were examined for cytotoxic activity 5 days later on the indicated target cells.

‡ i.v. inoculation of 1,000 HAU of inactivated virus or 100 HAU of infectious virus.

§ As in Table I.

‖ Values are the means ± standard errors of the mean from four wells with spontaneous release substracted.

tions prompted a re-examination of the capacity of UV-inactivated virus to stimulate in vivo secondary CTL responses under conditions where circulating anti-viral antibody was eliminated. This situation was achieved by adoptive transfer of spleen cells from mice primed with infectious A/WSN or A/JAP viruses into sublethally irradiated syngeneic recipients. Recipient mice were then challenged with either inactivated A/WSN, inactivated A/JAP, or the infectious homologous virus used in primary immunization. 5 days later, the spleens of these recipients were examined for cytotoxic activity (Table V). In contrast to the results obtained in vitro, recipients of A/JAP-primed spleen cells failed to respond either to inactivated A/JAP or A/WSN virus. Likewise, recipients of A/WSN primed spleen cells failed to generate cytotoxic responses when secondarily stimulated with either inactivated virus preparation. On the other hand, recipients of virus-primed cells, when inoculated with infectious homologous virus, generated significant CTL responses. Thus, in the absence of

TABLE VI
Sensitization of Target Cells for Cell-Mediated Cytotoxicity by Inactivated Influenza Virus

Target cell treatment*	Relative virus concentration‡	% Specific ^{51}Cr release from treated target cells§
A/JAP-Inactivated	2	23.7 ± 0.3
	20	20.7 ± 1.1
	200	19.9 ± 1.0
A/JAP-Infectious	1	86.2 ± 0.7
A/WSN-Inactivated	2	17.3 ± 0.3
	20	23.3 ± 0.8
	200	21.9 ± 0.7
A/WSN-Infectious	1	97.2 ± 1.1
B/LEE-Infectious	1	20.4 ± 0.6
None	–	16.5 ± 0.6

* ^{51}Cr-labeled P815 cells were incubated with the indicated virus preparation as described (Materials and Methods).

‡ Target cells were incubated either with infectious A/WSN, A/JAP, or B/LEE virus at a concentration of 30 HAU per 10^6 cells (10 EID_{50} U/cell) or with 2, 20, or 200-fold higher concentrations of inactivated virus.

§ Target cells were exposed to potent secondary cytotoxic effectors for 8 h at an effector:target ratio of 5:1. Cytotoxic effectors directed to influenza A/JAP were generated in vitro as described (Materials and Methods).

circulating anti-viral antibody, a potent CTL response can be obtained on secondary stimulation with homologous infectious virus. However, neither homologous virus nor heterologous type A influenza virus, when inactivated, stimulated a cytotoxic response under these conditions.

Lack of Target Cell Sensitization with Inactivated Influenza Virus. Several laboratories have reported that treatment of uninfected cells with inactivated paramyxoviruses (12, 13) or paramyxovirus subviral components (17) rendered these cells susceptible to specific lysis by cytotoxic T cells directed to these viruses. Because of the disparity between the in vivo and in vitro results described above, it was of interest to determine if inactivated influenza virus could sensitize putative target cells for lysis by influenza specific CTL in vitro. ^{51}Cr-labeled uninfected P815 mastocytoma cells were incubated under standard conditions (see Materials and Methods) with infectious A/WSN, A/JAP, or B/LEE viruses or with various concentrations of UV-inactivated A/WSN or A/JAP viruses and exposed in a standard cytotoxicity assay to highly potent influenza A/JAP-specific secondary effectors generated in vitro (Table VI). As demonstrated previously (Table IV and [19]), target cells infected with either A/WSN or A/JAP infectious virus were highly susceptible to lysis by these effector cells. On the other hand, target cells treated with inactivated A/WSN or A/JAP at concentrations up to 200-fold higher than the concentration of infectious virus needed to sensitize target cells, showed no lysis above background observed on B/LEE-infected or uninfected target cells. Identical results were obtained with secondary effectors specific for influenza A/WSN (not shown). The high degree of background lysis demonstrable on influenza B/LEE-infected or uninfected target cells has been previously observed with virus-specific cytotoxic T cells generated in vitro (3, 8) and was somewhat magnified by the relatively high effector to target ratio (5:1) and the longer incubation time (8 h) employed in the assay. These assay conditions were chosen to increase the possibility of detecting sensitization of target cells by inactivated virus.

Discussion

In this report, we have examined the issue of whether infectious virus is necessary both for the induction of CTL responses (i.e., stimulator cell sensitization) and for target cell sensitization or alternatively, whether induction and target cell sensitization can be achieved with noninfectious virus preparations. We have observed that while infectious influenza virus was highly efficient at inducing both primary and secondary influenza-specific CTL responses, noninfectious (UV-inactivated) influenza virus failed to stimulate detectable primary or secondary CTL responses in vivo. Similarly, noninfectious virus failed to sensitize target cells for lysis by influenza-specific cytotoxic T cells in vitro. However, inactivated virus could stimulate an influenza-specific secondary CTL response in vitro.

Before considering possible interpretations and implications of these results, two critical issues pertinent to our in vivo observations must be considered. The first issue is whether the inactivation procedure itself rendered the virus immunologically inactive. This possibility is unlikely since inactivated virus was capable of stimulating an adequate humoral immune response in vivo and could in vitro stimulate a specific cell-mediated cytotoxic response. The second issue is whether the parenteral administration of infectious influenza virus generates a sufficient antigen dose, as a result of replication in vivo, to induce a CTL response, whereas noninfectious (inactivated) virus fails to achieve such stimulatory antigen concentrations. We have attempted to approach this issue by examining the antigen dose dependence of CTL generation with both infectious and inactivated virus. Although low doses of infectious influenza virus (10^{-3}-10^{-2} HAU) induced detectable cytotoxic T-cell responses in vivo, no specific cytotoxic activity was detectable in vivo with 10^{5}-10^{6}-fold higher concentrations of inactivated virus (Fig. 1, Table I). Furthermore, since the humoral immune response to both infectious and inactivated virus was proportional to the immunizing virus dose and similar in magnitude, it is unlikely that extensive virus replication occurs in vivo after intravenous inoculation of infectious influenza virus. Also, current evidence indicates that the relevant target organs, presumably involved in the clearance of the parenterally administered virus inoculum (e.g., liver, spleen, lymph nodes), are not productively infected with influenza virus (26, 27). Taken together, these observations suggest that the difference between infectious and noninfectious influenza viruses in their respective capacities induce CTL responses in vivo is not purely a function of antigen dose in vivo.

A number of laboratories have recently reported results different from those reported here (12–15, 17). The results which are perhaps most germane to the present discussion involve the paramyxovirus model where inactivated virus preparations have been shown to both induce virus-specific CTL responses (12, 13) and sensitize target cells for T-cell-mediated lysis in vitro (12, 13, 17). The difference between these observations and those reported here, we believe, lies in the fact that the paramyxovirus virion possesses a specific fusion protein (28) which allows for the efficient integration of virion surface antigens into the cell cytoplasmic membrane (28) and also promotes cell-to-cell fusion (29). Such fusion activity has not been demonstrated in influenza viruses (29, 30). Furthermore, it has been recently reported that a functionally active fusion protein is necessary for the sensitization of target cells by a UV-inactivated paramyxovirus (31). In the light of these findings and our inability to sensitize target cells with inactivated influenza viruses (Table VI), we propose that both for the induction of virus-specific CTL responses and for the expression of the effector activity of CTL, the relevant viral antigens must be presented on the surface of the putative stimulator or target cell as integral membrane components, i.e., inserted into the membrane lipid bilayer. Such a situation could be readily achieved either by direct integration of the virion antigens into the cell membrane through fusion, as in the case of paramyxoviruses and other viruses which possess efficient fusion capacity or as exemplified by viruses such as influenza, which lack such efficient fusion activity, by incorporation of nascent antigens into the cell membrane during the course of virus infection.

Although our results on the induction of CTL responses in vivo and target cell sensitization in vitro with inactivated influenza virus are consistent with the above hypothesis, the induction of a secondary cytotoxic response to inactivated virus in vitro is in apparent disagreement. This result is open to two interpretations: first, it is possible that there is a qualitative difference in the requirements for CTL induction under in vitro conditions of stimulation, i.e., precursors of cytotoxic T cells can be directly stimulated by free virus or virus adsorbed to the stimulator cell surface in vitro but not in vivo. Second, it is possible that this difference is quantitative, i.e., inactivated influenza virus is capable of sensitizing stimulator cells by integration of virion antigens into the cell cytoplasmic membrane but with an efficiency too low to be detectable either at the level of target cell sensitization in vitro or under in vivo conditions of stimulation. In vitro conditions of stimulation, on the other hand, would favor the detection of a response to the small number of sensitized stimulator cells generated by inactivated virus. A resolution of this point may come from experiments with purified influenza virus antigens which have recently been shown to stimulate a specific CTL response from primed cells in vitro (32).[2] Such studies are now in progress.

At least two distinct subpopulations of cytotoxic T cells are generated in response to infectious type A influenza virus (19, 24), one of which is specific for the immunizing virus strain (virus-strain-specific), the other of which exhibits a high degree of crossreactivity for target cells infected with type A influenza viruses of any subtype (19, 24, 33). Current evidence suggests that the target antigens for these two cytotoxic subpopulations are the influenza virion surface glycoproteins (hemagglutinin and possibly neuraminidase) and the internal virion antigen matrix protein, respectively (34). Since inactivated influenza A/JAP stimulated a cytotoxic response which was specific for A/JAP targets (Table IV), only the virus-strain-specific CTL subpopulation appears to have been generated in response to this virus preparation.[3] This result implies that the in vitro response to inactivated A/JAP virus was not due to a low level of infectious virus in the virion preparation since infectious A/JAP virus also stimulates a response in the crossreactive cytotoxic subpopulation (Table IV). Zweerink et al. (32), however, have recently reported the induction of a CTL response to UV-inactivated influenza virus which was highly cross-reactive. The most likely cause for the discrepancy between our results and those of Zweerink et al. is the difference in the extent of virus inactivation: 7 min vs. 1 min UV exposure, respectively. Since the infectivity of an influenza virus preparation is lost more rapidly during inactivation than the capacity of the virions to direct the synthesis of specific viral antigens (35), it is possible that a partially inactivated virus preparation, although incapable of producing infectious virions, is capable of directing the synthesis and expression of relevant viral antigens on the stimulator cell surface during an abortive cycle of replication. Consistent with this concept is the observation that the putative target antigen for cross-reactive cytotoxic subpopulation, influenza matrix protein (34) is expressed on the cell surface during the course of infection (34, 36, 37) but is internally located in the influenza virion (30). Two other reports of CTL responses to inactivated virus (14, 15) might be explained on a similar basis, i.e., synthesis and expression of the relevant viral antigens in the absence of infectious virus production. Since, in these reports, the capacity of inactivated virus to sensitize target cells or to direct nascent viral protein synthesis was not examined, the discrepancy between these observations and those reported here, remains to be resolved.

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[2] T. J. Braciale, and T. J. Higgins. Manuscript in preparation.

An observation reported here which warrants further discussion is the capacity of inactivated influenza virus to stimulate an in vivo primary humoral immune response in the absence of a detectable CTL response in vivo. Since the induction of an in vivo primary humoral immune response to influenza virus has been shown to be thymus-dependent (38–40), it is likely that helper T cells can be activated by either infectious or inactivated influenza virus in vivo. However, only infectious virus stimulates a detectable CTL response in vivo. One possible interpretation of this observation is that helper T cells and cytotoxic T-cell precursors differ in their requirements for induction with respect to mode of antigen presentation. According to the hypothesis outlined above, the induction of a CTL response to specific viral antigens would require their presentation on the stimulator cell as integral membrane components, whereas the activation of helper T cells directed to these antigens could be achieved in a manner analogous to that suggested for soluble antigens (41). In this connection, it should be noted that precursors of helper T cells and precursors of cytotoxic T cells also differ in their requirements for induction with respect to genes in the MHC (42–44). The question of whether this genetic difference in the requirement for helper and cytotoxic T-cell induction is a reflection of the difference in the mode of antigen presentation will require further analysis of these two T-cell subsets.

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[3] By using appropriate recombinant influenza virus strains, the specificity of the cytotoxic cells for influenza A/JAP hemagglutinin has been demonstrated (T. J. Braciale, unpublished observations).

The requirement for viral infectivity in the induction of virus-specific cytotoxic T cells remains to be fully elucidated. Factors which should be considered in assessing this requirement include: (*a*) whether the virus employed has efficient fusion activity which could promote efficient integration of virion antigens into the cell cytoplasmic membrane; (*b*) whether an inactivated virus preparation which fails to undergo a complete cycle of replication, i.e., produce infectious virus, is also incapable of inducing nascent viral protein synthesis during an abortive cycle of replication; (*c*) whether the analysis is undertaken in vivo or in vitro. Based on our own observations and those of other investigators we have proposed that both the induction of virus-specific cytotoxic T cells and the expression of their lytic activity requires the presentation of the relevant viral antigens on the surface of the stimulator or target cell as integral membrane components. Experiments are now in progress to test this proposal.

Summary

This report examines the requirement for infectious virus in the induction of influenza virus-specific cytotoxic T cells. Infectious influenza virus was found to be highly efficient at generating both primary and secondary cytotoxic T-cell response in vivo. Inactivated influenza virus however, failed to stimulate a detectable cytotoxic T-cell response in vivo even at immunizing doses 10^5–10^6-fold higher than the minimum stimulatory dose of infectious virus. Likewise inactivated virus failed to sensitize target cells for T-cell-mediated lysis in vitro but could stimulate a specific cytotoxic response from primed cells in vitro. Possible requirements for the induction of virus-specific cytotoxic T-cell responses are discussed in light of these observations and those of other investigators.

The authors wish to thank Professor G. L. Ada for helpful discussions and encouragement, and Professor Ada, Dr. V. L. Braciale, and Dr. R. V. Blanden for critical review of this manuscript. The excellent technical assistance of S. Henty and the excellent typing of L. Hardy are gratefully acknowledged.

Received for publication 19 December 1977.

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PROLIFERATIVE CAPACITY OF MOUSE PERITONEAL MACROPHAGES IN VITRO*

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Intraperitoneal injection of thioglycolate medium or other phlogogenic stimuli into mice or rats will increase the number of mononuclear phagocytes severalfold within a few days (1–3). This is due to an invasion of newly formed cells originating from the bone marrow (1, 2, 4–6). Whereas normal "resident" macrophages rarely synthesize DNA and divide in vivo, "activated" macrophages will synthesize DNA and proliferate.

In the presence of L-cell-conditioned medium, activated macrophages can also be induced to proliferate in vitro (7) and, under appropriate culture conditions, will produce colonies of mononuclear phagocytes with a relatively high plating efficiency (3, 8). The component in the L-cell-conditioned medium that induces this response is a glycoprotein of about 60,000 daltons, referred to as macrophage growth factor (MGF; 9–11).[1] In contrast, resident mononuclear phagocytes cannot be induced to proliferate in vitro (3, 8).

Activated macrophages also differ in many other respects from resident macrophages. Activated macrophages (*a*) have greater pinocytic activity (12); (*b*) show enhanced phagocytosis (13); (*c*) spread out and attach more firmly to wettable surfaces (14); (*d*) spontaneously synthesize and excrete neutral proteases like collagenase (15), elastase (16, 17), and plasminogen activator (18–20); and (*e*) have a lower membrane bound 5′-nucleotidase activity (21). These differences suggest that activated macrophages represent a cell population distinct from resident macrophages.

In this study, we sought to determine whether mononuclear phagocytes could be subcultured, how many times cells could be passaged and still maintain exponential proliferation, the average number of progeny a single precursor cell could produce, and the role of MGF in the regulation of their proliferation. In addition, we used 5′-nucleotidase activity as a marker for activation to determine whether senescent macrophages, exhausted in their proliferative capacity,

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* Supported by grant 5P01CA13053-05 awarded by the National Cancer Institute, Department of Health, Education, and Welfare, and grant Al 12242 from the National Institute of Allergy and Infectious Diseases.

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[1] *Abbreviations used in this paper:* CFC, colony-forming cells, clonogenic cells; MEM, Eagle's minimum essential medium; MGF, macrophage growth factor.

J. EXP. MED. © The Reckefeller University Press · 0022-1007/78/0401-1253$1.00

behaved like resident nonactivated macrophages. These studies have both practical and theoretical importance. A single progenitor cell must be capable of producing a sufficient number of progeny if functional studies, such as those mentioned above, are to be carried out on individual colonies. Using these cells, we can directly address questions concerning the functional heterogeneity of mononuclear phagocytes. Knowledge of their proliferative capacity would also contribute to our understanding of this cell system's ability to expand in the host defense to infections and malignant growth.

Materials and Methods

Macrophages. Cells were obtained from 11- to 16-wk-old C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, Maine) as described before (3). Briefly, mice were injected intraperitoneally with 1.5 ml Brewer's thioglycolate medium (Difco Laboratories, Detroit, Mich.) 72 h before harvest. Cells were collected after intraperitoneal injection of 5 ml α-MEM (Eagle's minimum essential medium [22]; Flow Laboratories, Inc., Rockville, Md.) medium supplemented with 10% fetal calf serum and 5 U heparin/ml, and were washed once with the same medium without heparin. Growth medium consisted of α-MEM, supplemented with 10% L-cell-conditioned medium. For cell counts, the cells were lysed by cetrimide, and nuclei were counted with an electronic particle counter (23). Pronase digestion of dead cells was omitted because detachment is concomitant with cell death.

Conditioned Medium. L-cell-conditioned medium was used as the source of MGF for most experiments. We prepared large batches by plating 175 ml of L cells at 5 × 10^4 cells/ml in 1,300 cm^2 roller bottles. After 4 days the culture medium (α-MEM supplemented with 10% fetal calf serum) was collected.

To prepare fibroblast-conditioned medium, we obtained embryo fibroblasts from 14- to 19-day-old fetuses and established cultures at 5 × 10^5 viable cells per milliliter (10 ml) in replicate 100-mm tissue culture dishes. The fibroblast-conditioned medium was harvested 7 days later.

Culture Conditions. Cells were grown in a humidified incubator having a 5% CO_2 atmosphere. Unless otherwise noted, cells were plated in 35-mm plastic tissue culture dishes containing 3 ml growth medium. If necessary, parallel 100-mm dishes were plated with the same cell suspension and the same volume to surface ratio (25 ml per dish).

To determine the plating efficiency, we established cultures, derived from either the primary cells or the removed subcultured cells plating 1,000 cells in 3 ml growth medium on 35-mm dishes. After 14 days incubation, we stained these cultures and counted the number of colonies, defining "colony" as a cluster of 50 or more cells.

When unstimulated macrophages were used, the nonadherent cells (about 75%) were removed by rinsing the plates repeatedly with medium 2 h after the cells were plated.

Phagocytosis Assay. Phagocytosis of autoclaved bakers' yeast in the presence of guinea pig complement has been described (3). Briefly, 5 × 10^7 yeast particles and guinea pig complement (3% final concentration) were added to culture dishes. After 30 min incubation at 37°C, the dishes were rinsed with phosphate-buffered saline and stained with methylene blue.

Pulse Labeling with [^{3}H]Thymidine and Autoradiography. Cells grown in dishes having an 18 × 18-mm sterile coverslip were supplemented with 5 μCi/ml [methyl-^{3}H]thymidine (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y., 58 Ci/mmol). After 2 h, the coverslip was removed, washed once with phosphate-buffered saline, twice with 5% trichloroacetic acid, and once with ethanol:ether 1:1 by volume. The dry coverslips were mounted onto microscope slides, dipped into NTB-2 liquid photographic emulsion (Eastman Kodak Co., Rochester, N.Y.), and exposed for 3-4 wk before developing. After staining with Giemsa, we determined the fraction of labeled cells (labeling index).

Subculturing of Macrophages. Cells were incubated at 37°C for 30 min. in medium supplemented with 1/6 vol of 2% lidocaine-HCl (Astra Pharmaceutical Products, Inc., Framingham, Mass.), giving a final concentration of 12 mM (24, 25). We used 2 ml of medium for 35-mm dishes and 5 ml for 100-mm dishes. The cells rounded up during this incubation period and were removed by a jet of medium from a Pasteur pipette. The cells were diluted 10-fold with α-MEM supplemented with 10% fetal calf serum; they were then collected by centrifugation (200 *g* for 5 min), resuspended in growth medium, counted, appropriately diluted, and replated.

5'-Nucleotidase Assay. We adapted the method used by Edelson and Cohn (21) to assay the activity in a smaller number of cells. The cells in a 35-mm culture dish were rinsed twice with serum-free medium and then lysed with 1 ml buffer solution (54 mM Tris-Cl, pH 9.0, 12 mM $MgCl_2$, 0.05% Triton X-100). A volume of 0.1-0.5 ml of this lysate was incubated at 37°C in a final volume of 0.7 ml buffer solution containing 5×10^{-5} M unlabeled AMP and 8.4×10^{-10} M (0.014 μCi/ml) [^{3}H]AMP (adenosine-[2-^{3}H]-5'monophosphate, ammonium salt, 17 Ci/mmol, obtained from Amersham/Searle Corp., Arlington Heights, Ill.). The reaction was terminated by adding 0.25 M solutions, 0.15 ml each of $ZnSO_4$ and $Ba(OH)_2$. Unhydrolyzed AMP and $BaSO_4$ coprecipitate under these conditions. We removed this precipitate by centrifugation (10,000 *g* for 10 min) and determined the amount of adenosine that had been formed by counting 0.5 ml of the supernate in 3 ml xylene-Triton X-117 scintillation fluid (26). The amount of adenosine formed after 1- and 2-h incubation periods was plotted, and the reaction rate was calculated from the slope of this curve.

Enzyme levels were expressed as nanomoles per minute per 10^6 cells; this number of cells corresponds to 238.3 ± 43.0 μg protein determined by the Lowry method (27) with bovine serum albumin as a standard.

Results

Growth Kinetics of Primary Cultures of Thioglycolate-Stimulated Macrophages. Under our present culture conditions, when macrophages were plated at cell concentrations of approximately 3,000 cells/ml or less, the number of cells doubled every 40 h (Fig. 1 A, closed points), somewhat faster than previously reported (3). The duration of exponential growth, however, was dependent on the initial cell concentration, and cells entered plateau phase when the cell number reached 2-3 × 10^5 cells per culture plate (35 mm). These cultures were not completely confluent, and if the cells were maintained for a longer time in culture many rounded up and detached from the plate. Within 4 days of growth, the fraction of cells in S-phase during a 2-h [^{3}H]thymidine pulse labeling reached over 30% (Fig. 1 B, closed points). This labeling index remained relatively constant during the exponential growth period and then dropped abruptly as cells entered stationary phase.

Synthesis of MGF by Contaminating Fibroblasts. When peritoneal cells are obtained, they are invariably contaminated by a small number of fibroblasts; we usually find 1 colony-forming fibroblast per 1,000 exudate cells (0.1%) or about 1 colony-forming fibroblast per 50 colony-forming macrophages (2%). Because fibroblasts can produce MGF, they could, in time, produce enough endogenous factor to stimulate the macrophage precursors to proliferate, provided that the precursors had survived that long.

To determine the survival of the macrophage colony-forming cells (CFC), we plated peritoneal exudate cells into 35-mm culture dishes at 1,000 cells per dish in 3 ml α-MEM containing only 10% fetal calf serum and 5% horse serum. Fibroblast-conditioned medium was added to replicate dishes on days 0, 1, 4, 7, or 14 to a final concentration of 10%; the number of colonies was determined 14 days later. The results are shown in Table I. No significant difference was found in the number of colonies formed as a function of the time at which the conditioned medium was added. Thus, the CFC survive in culture as resting cells for at least 14 days and can be induced to proliferate when the conditioned

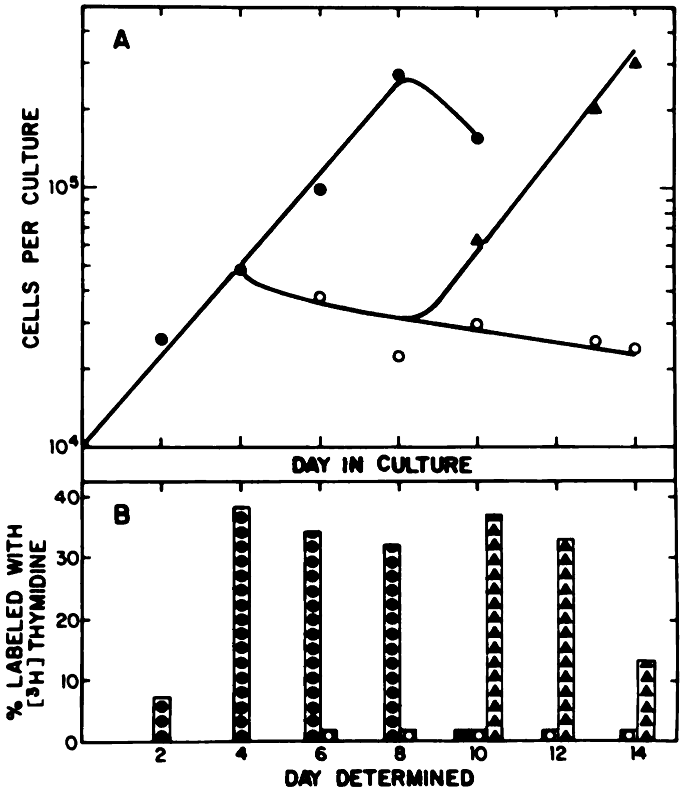

FIG. 1. Dependence of macrophages on the continuous presence of MGF for cell division and DNA synthesis. (A) Macrophages were grown in the continuous presence of MGF (●). On day 4, the medium of some plates was removed, the cells were washed and refed with medium lacking MGF (○). On day 8, the medium removed on day 4 was readded to the cells (▲). (B) Parallel plates were pulse labeled with [^{3}H]thymidine; the fraction of the labeled cells was then determined after autoradiography. Phagocytosis on other parallel plates was done on days 4, 8, and 12. In all cases >99% of the adherent cells phagocytized yeast particles.

TABLE I

Macrophage Colonies Induced by Fibroblast-Conditioned Medium

Day FCM* added	Assay	Macrophage colonies per 1,000 cells‡	
		With FCM	Without FCM
	day		
0	14	42 ± 12§	0
1	15	48 ± 35	0
4	18	54 ± 23	0
7	21	35 ± 10	0.7 ± 0.6
14	28	48 ± 6	6 ± 5

* Fibroblast-conditioned medium.

‡ The number of contaminating fibroblast colonies, all groups, was 0.97 ± 0.8 per 1,000 cells.

§ 1 SD.

TABLE II
Production of MGF by Fibroblasts

Day FCM* obtained	Macrophage colonies per 1,000 cells
2	1 ± 4‡
4	21 ± 30
7	74 ± 62
9	95 ± 83
14	71 ± 98

* Fibroblast-conditioned medium (10%) was added to α-MEM supplemented with 10% fetal calf serum and 5% horse serum.
‡ 1 SD.

medium is added. Table I also shows that the number of macrophage colonies in culture increases after 21 days in the absence of conditioned medium.

To test whether the contaminating fibroblasts produce enough MGF to stimulate some of the CFC to proliferate, we established cultures containing 10,000 embryo fibroblasts in 3 ml medium. After 2, 4, 7, 9, or 14 days of incubation, medium was removed from replicate cultures of fibroblasts. After all media were collected, they were tested individually for their ability to stimulate macrophage proliferation. The results are shown in Table II. By day 7 the fibroblasts had produced enough MGF to maximally stimulate all the macrophage CFC, suggesting that contaminating fibroblasts are likely to provide this kind of stimulation. As direct proof that a single fibroblast colony can produce enough MGF to stimulate CFC to proliferate, Fig. 2 shows that visible colonies of macrophages form near a fibroblast colony but not at a distance from it. Because the expected frequency of fibroblast contamination (1/1,000 cells) is 63%, not all macrophage cultures are contaminated; when contamination does not occur, macrophage colonies never form; the latter possibility if also shown in Fig. 2, for comparison.

Control of Proliferation by MGF. To determine whether MGF is required continuously or acts merely as a trigger for cell division, the growth medium was removed on day 4 and replaced with culture medium containing only 10% fetal calf serum and 5% horse serum. The results, shown in Fig. 1, indicate that removal of MGF causes cells to enter a stationary phase; < 1% of these cells synthesized DNA 48 h later. In fact, by 12 h after removal of MGF, the percentage of labeled cells had fallen from 33 to 17% and by 24 h was only 0.5%. Cells surviving during stationary phase are able to phagocytize bakers' yeast but some cells become detached from the plates.

If growth medium is readded to the cultures 4 days later, the "resting" macrophages reenter the cell cycle as evidenced by an increase in the labeling index to 37% by day 10 (Fig. 1). This means that about the same fraction of cells is in cell cycle as just before removal of the MGF. It appears that not all the cells enter the cell cycle during the first 24 h because the maximum labeling index of 37% was not found until 48 h. Also by day 10, the number of cells increases again, at a rate equal to that found for the control cultures from which growth medium was not removed.

Results similar to those shown in Fig. 1 have been obtained with cells that had been deprived of MGF for longer intervals, up to 7 days. In each case, its

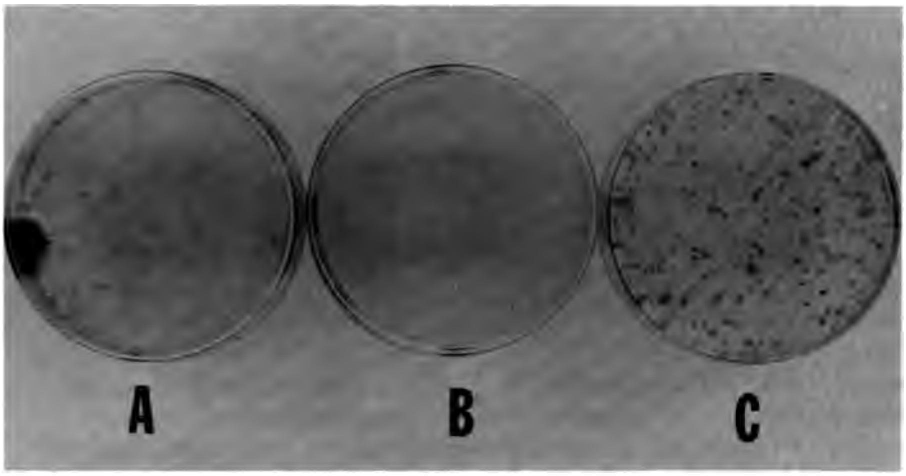

FIG. 2. Effect of fibroblast colonies on macrophage growth. In A, a fibroblast colony is shown at the lower left corner. The smaller colonies of macrophages are found around this colony, but not at a distance from it. In B, a similar culture dish is shown without any fibroblast or macrophage colonies, whereas in C, these same exudate cells are shown to have developed into macrophage colonies when MGF had been added.

readdition resulted in reentry of macrophages into the growth cycle. However, when the interval of time without the factor is long, an increasing number of macrophages were found to enter the cell cycle spontaneously, even though exogenous growth factor was absent. This was due to contamination of these cultures by fibroblasts that produce endogenous factors, as was shown in Table II and Fig. 2. This stimulation did not occur in cultures which, upon microscope examination, lacked fibroblasts.

Proliferative Capacity of Macrophages. When macrophages are cultured in 35-mm culture dishes, the cell number never exceeds 5 × 10^5 and generally reaches a plateau at 2-3 × 10^5. This represents about a 20% confluent monolayer of cells on a 35-mm culture dish. The failure of these cells to establish a confluent monolayer could be due to the limited proliferative ability of precursor cells, to a depletion of essential nutrients, or to some contact inhibitory phenomena within the colony. When cultures were initially established at 10^3, 5 × 10^3, or 10^4 cells per plate in 3 ml growth medium, the final cell numbers achieved were very much the same (compare the curves for primary macrophages in Fig. 3 A-C). Thus, it seems likely that depletion of essential nutrient(s) or contact inhibition is responsible for cell entrance into stationary phase. We have previously shown that peritoneal exudate cells completely deplete glucose in less than 48 h when cultured as a confluent monolayer (24). Thus, the cells must be subcultured at lower cell densities in order to evaluate their proliferative capacity.

Primary cultures of peritoneal exudate cells were established at either 10^4, 5 × 10^3, or 10^3 cells in 35-mm culture dishes. The cell number was determined frequently and when the level reached 1-3 × 10^5 cells per culture, the cells from a parallel 100-mm dish were removed with lidocaine, diluted, and replated onto 35-mm dishes for determining cell numbers and onto 100-mm dishes for eventual

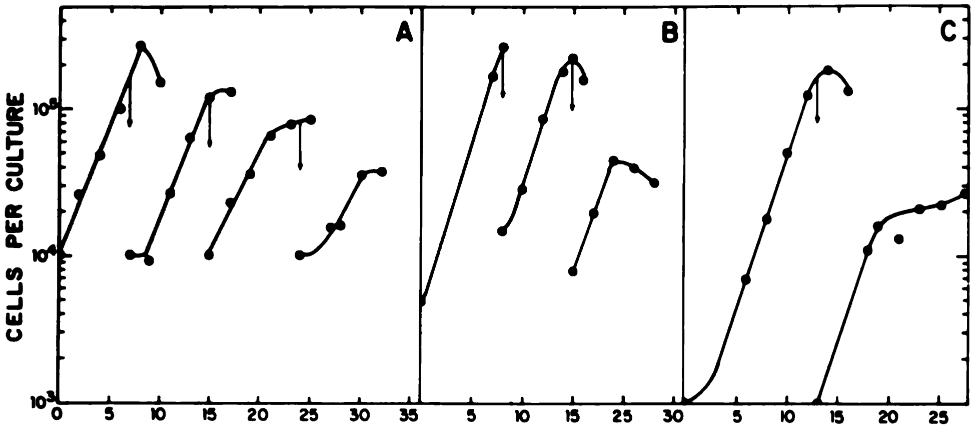

Fig. 3. Proliferative capacity of macrophages in vitro. Macrophages for cell counts were grown in 35-mm dishes at initial cell densities of: (A) 10^4; (B) 5×10^3; and (C) 10^3 cells per dish. At the times indicated by arrows, the cells of parallel 100-mm plates were treated with lidocaine and subcultured. A and C are experiments in which the same macrophages were used. Phagocytosis assays were carried out on days 4, 8, 12, 16, 20, 24, and 28. In all cases, >90% of the cells were phagocytic. Fibroblasts developing into colonies were found on some cultures.

subculturing. The results of this study are shown in Fig. 3. Subcultured cells continued to divide at the same rate as did the primary macrophages. The doubling time for the population was approximately 40 h. However, with each subculture, the final yield of cells decreases. A final cell density of < 20% of that achieved by the primary macrophages (5×10^4 cells per 35-mm dish) was assumed to mean that the cells had nearly exhausted their proliferative capacity.

The data suggest that the precursor cells can undergo only a limited number of cell divisions; however, this limited number is considerably greater than that attained in the original culture. The total number of cells that would have accumulated from subculturing all of the cells was 6.0×10^7 for primary cultures established at 10^4 cells per culture, an overall increase of 6,000 times. For an initial cell number of 5×10^3, the increase was 4,400-fold or 2.2×10^7 cells; for 10^3 cells, it was 4,600-fold or 4.6×10^6 cells. The average increase for all concentrations was 5,000 times the original cell number, and the degree of increase did not depend greatly on the number of cells cultured or the number of subcultures.

The plating efficiency for the primary cultures and for each subculture thereafter was determined to find out whether the fraction of CFC (presumed stem-line) decreased with subculture. The plating efficiency of the primary cells was found to be 20.1%. With each successive subculture, the plating efficiency decreased to 6.2, 2.6, and 1%, respectively. An initial plating efficiency of 20.1% implies that starting with 10^4 cells per culture, 2,010 CFC were able to produce 6×10^7 cells by the end of the third subculture. If we assume that all of these cells are derived from the initial clonogenic fraction, the average CFC underwent 14.9 divisions and produced 3×10^4 progeny. When the initial number was

10^3 cells per culture, the average CFC underwent 14.5 divisions and produced 2.3×10^4 progeny.

Every 4 days throughout these experiments we assayed parallel cultures for phagocytic activity to be sure that the proliferating cells were macrophages and not contaminating fibroblasts. In all cases, nearly every cell was phagocytic. Occasional fibroblasts were encountered on some plates but not on others; these fibroblasts were found in discrete localized colonies surrounded by macrophages and represented, at the most, 5% of the cells on the plate at the time stationary phase was achieved.

Lidocaine Removal of Macrophages. If not all of the cells survive the preparative procedure for subculturing, the proliferative ability of macrophages might actually be greater than that described above. To evaluate what fraction of the cells recovered with lidocaine is viable, primary cultures of macrophages were treated with lidocaine at different times after plating: 1, 4, and 23 h and 7 and 8 days. The removed cells were washed and then resuspended in their original volume of medium. Samples of 3 ml were replated, and either 1 or 24 h later we determined the number of cells that would reattach. To compare the plating efficiency of these cells to that of untreated cells, we established cultures of 10^3 cells in 3 ml growth medium. Colonies were counted 14 days later. For this experiment 10^6 cells were initially plated onto 35-mm dishes in 3 ml α-MEM containing only 10% fetal calf serum for the 1-, 4-, and 23-h determinations. For the 7- and 8-day assays 10^4 cells were plated onto 35-mm cultured dishes in complete growth medium. All plates were washed 1 h after plating to remove nonadherent cells.

As shown in Table III, 75–94% of the cells could be removed with lidocaine with an overall recovery, after centrifugation, of between 58 and 77%. Table III (column 5) shows the recovery of viable macrophages after they were allowed to reattach for either 1 or 24 h. The results were surprising. When primary cultures are treated with lidocaine during the first 24 h, the overall recovery of viable macrophages is very poor, between 5 and 11%. However, if these cells are cultured in growth medium 7–8 days and then treated with lidocaine, the recovery of viable cells improves to around 40%. This same pattern is reflected in the number of CFC (columns 6 and 7). We included the 24-h determination of attached cells in this study because cells treated with lidocaine might require a period of time to recover and, therefore, might not attach as quickly as untreated cells. Alternatively, the treated cells, although viable soon after treatment, may have accumulated lethal damage which requires time for expression. The latter effect appears to be significant for freshly obtained macrophages, because reattachment after 1 h incubation was greater than that after 24 h.

5′-Nucleotidase Activity of Macrophages. The activation state of proliferating macrophages in culture is not known. One possibility is that when macrophages reach proliferative senescence they might behave like normal, unstimulated, "resident" macrophages. Edelson and Cohn (21) have shown that the membrane-bound enzyme 5′-nucleotidase can be used as a probe for macrophage activation. "Activated" macrophages have a low enzyme activity, whereas "resident" macrophages have a rather high enzyme activity. Our

TABLE III

Viability of Primary and Cultured Macrophages Treated with Lidocaine

Time of treatment*	% Recovered‡		Assay time	Percentage attached viable cells§	Plating efficiency after lidocaine treatment	
	Removed from plate	After centrifugation			Colonies‖ / 1,000 cells	Percentage of control
			h			
1 h	85	70	1	18	1 ± 0.82	0.7
			24	5	–	
4 h	75	58	1	17	3.62 ± 1.01	2.4
			24	6	–	
23 h	94	77	1	12	9.3 ± 1.01	6.2
			24	11		
8 days	88	–	–	–	74	49
7 days	81	75	1	45	60	40
			24	58		
7 days	81	66	1	39	52	35
			24	39		

* This is the time after cells were placed in dishes.

‡ The fraction of macrophages removed from the original plate was determined by dividing the number of cells removed by the number of attached cells on a replicate untreated plate. This was verified by also counting the number of cells left behind on the lidocaine-treated plate.

§ The percentage reattachment was calculated from the ratio of the number of cells that reattach to a culture dish to the number of cells on the original culture dish before treatment with lidocaine. This figure represents the yield of viable macrophages.

‖ The number of colonies on untreated control plates was 150 ± 10 per 1,000 cells.

results confirm their findings. We compared unstimulated and thioglycolate-stimulated macrophages that had been cultured for 24 h in α-MEM with 10% fetal calf serum. We used 35-mm dishes containing about 5×10^4 cells in 3 ml medium. The unstimulated macrophages had a 5′-nucleotidase level of 14.35 ± 0.35 nmol/min per 10^6 cells. In thioglycolate-stimulated cells we could not detect any enzymatic activity. However, when these same cells were allowed to proliferate in the presence of MGF, they acquired detectable enzyme levels. For instance: 10^4 cells divided in 4 days to about 5×10^4 cells. These logarithmically growing cultures had an enzymatic activity of 4.4 nmol/min per 10^6 cells or 31% of the value found for resident macrophages. Table IV shows the 5′-nucleotidase levels of these macrophages and their subcultured progeny, determined every 4 days. At first sight the enzyme levels fluctuate randomly between 3 and 44% of the value found for resident macrophages. A more careful look at the growth kinetics of these cells (see Fig. 3) indicates that enzyme levels are high in logarithmically growing cells and lower when cultures reach the stationary phase. There is no indication that the cells acquire enzyme levels similar to those found in resident macrophages when they have nearly exhausted their proliferative capacity.

Discussion

This study shows that macrophage precursors obtained from the peritoneal

TABLE IV

*5'-Nucleotidase Activity of Macrophages Proliferating in Vitro**

Day‡	10^4 Cells plated per dish		10^3 Cells plated per dish	
	5'-Nucleotidase	Subculture	5'-Nucleotidase	Subculture
	nmol/min/10^6 cells		*nmol/min/10^6 cells*	
4	4.4	0	–	
8	1.4	0	6.3	0
13	3.4	1	0.9	0
16	0.8	1	–	
16	1.5	2	–	
20	2.0	2	0.8	1
24	0.5	2	0.5	1
28	2.6	3	–	

* The cultures were replicates of those used in the experiments reported in Fig. 3.

‡ After establishing the initial culture.

cavity of thioglycolate-stimulated mice have an impressive, though limited, proliferative capacity. Our average yield of peritoneal exudate cells after thioglycolate stimulation is 8×10^6 cells or 1.6×10^6 clonogenic cells per mouse (20%). Inasmuch as the average number of progeny is 2.6×10^4 per CFC, the exudate cells from a single mouse could potentially yield a total of 4.2×10^{10} cells. As the mean cell volume was 983 μm^3 (unpublished observations), the total progeny from a single mouse would yield a packed cell volume of about 40 ml of macrophages after 30 days of proliferation. Clearly, this proliferative potential is not fully realized in vivo during the inflammatory response, and macrophage proliferation must be under some type of control that is lost when they are transferred in vitro.

The results of our studies show that a positive stimulus (MGF) must continuously be present to maintain macrophages in a growth cycle. Removal of the stimulus causes these cells to return to a nonproliferative "resting" state; a state that can be entirely reversed by the readdition of the stimulus. Other hemopoietic cells seem to be similarly regulated: for example, the formation of granulocyte and macrophage colonies by bone marrow cells in the presence of colony stimulating factor (10), and the differentiation and division of erythroid cells to hemoglobin-producing cells by erythropoietin (28). Also the lymphocyte proliferative response requires the continuous presence of mitogens (29, 30).

The results shown in Table III indicate that even though lidocaine yields more viable cells when it is used to remove cultured macrophages than when it is used to remove freshly obtained cells, still more than half of the former cells do not survive the treatment. Cultured macrophages have been growing in culture and they have been bathed in medium containing 10% fetal calf serum, 5% horse serum and 10% L-cell-conditioned medium. Only further exploration will reveal whether either or both of these variables affect the viable cell recovery after lidocaine treatment. Even with the relatively poor recovery of viable macrophages, their proliferative ability is quite extensive and might have been even greater had they all been removed in a viable state for subculture.

The plating efficiency dropped from 20% for primary macrophages to 1% for quaternary cultures. As shown by our viability studies after lidocaine treatment, we can account for at least part of the decrease in plating efficiency by the failure to recover all of the cells in a viable state for subculture. However, this cannot explain the observation that the maximum density, achieved when cells enter stationary phase, decreases after each subculture. Because subculture results in further proliferation with a doubling time similar to that of the original cell population (Fig. 3), it might be expected that cells, as long as they have remaining proliferative capacity, will continue to proliferate up to the same culture density of $2-3 \times 10^5$ cells per plate. The fact that these cells do not achieve this level suggests that each colony must reach some maximum size at which macrophages cease to proliferate though potentially capable of more divisions. Not until the individual colony is disrupted and the cell density reduced by subculture are the cells again capable of proliferation, albeit a smaller proportion of them. One possible explanation for this phenomenon is contact inhibition within the colony. Although we have no direct evidence that this is the cause, such a hypothesis would explain the continual reduction in total cell yield. Alternatively, the failure of the macrophages to continue to proliferate may be an artifact introduced by the lidocaine treatment.

We have referred to the cells from a thioglycolate medium-elicited exudate as activated macrophages. When the most recent classification for mononuclear phagocytes was proposed (31), there was no need to provide for cells with self-replicating properties not localized in the bone marrow. Clearly, we and others (3, 7-9) have accumulated ample evidence to show that these peripheral cells can replicate. Therefore, the proposed classification should be changed to include these precursor cells which, though derived from the bone marrow, retain an extensive self-replicating potential when they infiltrate peripheral tissues.

Although current thought favors the concept that activated peritoneal macrophages induced by agents like thioglycolate medium are derived from the bone marrow via the blood, the source of the resident macrophage is not as clear; these resident macrophages might be the senescent progeny of activated macrophages. It has been our thought that once cultured activated macrophages have exhausted their proliferative capacity, they might behave like resident, nonactivated macrophages. Thus 5′-nucleotidase activity was chosen as a probe for the state of activation of cultured macrophages. The results of our studies on this point are somewhat equivocal. Even though exponentially growing macrophages showed higher 5′-nucleotidase activity than did their nonproliferating, newly derived counterparts, they never acquired the levels found for resident macrophages, nor did subculture produce any trend in that direction. Lidocaine treatment cannot be overlooked as having an influence on the results. Nevertheless, these macrophages are a mixture of proliferating and nonproliferating cells, and this population could be expected to have a lower activity than one predominantly composed of resident macrophages. It is clear that further work will be necessary before any firm conclusion can be derived.

Because activated macrophages can be cultured easily and can be made to proliferate or rest at will, they lend themselves to further study of the part of the cell cycle where the decision is made to continue to proliferate or not. It is unlikely that removal of the growth factor results in immediate cessation of all events involved in proliferation. Rather, cells that have progressed past the point of decision proceed through cell cycle. By using this system as an example, it may be possible to look more precisely at the events that occur when the decision is made. Their proliferative capacity can also be exploited to amplify single clones of macrophages into sufficient numbers for studies dealing with macrophage function. These clones would be absolutely uncontaminated by other cells because they would have been derived from a single isolated precursor cell.

Summary

Thioglycolate-stimulated mouse peritoneal macrophages cultured in the presence of macrophage growth factor (MGF) will continue to proliferate when they are removed from culture dishes with the local anesthetic lidocaine and subcultured. The number of times the cells can be subcultured and remain in a proliferative state is dependent on the number of previous cell divisions. One precursor cell (colony-forming cell) yields about 2.6×10^4 daughter cells.

When MGF is removed from actively proliferating macrophages, they leave the cell cycle and enter a "resting" condition. When MGF is readded, cells reenter the cell cycle and proliferate with the same doubling time as if MGF had not been removed.

Membrane 5′-nucleotidase activity was used as a probe to identify the state of macrophage activation. Proliferating macrophage populations had significantly higher enzyme levels than stimulated macrophages cultured without MGF. These enzymes levels were, however, lower than those found for resident (unstimulated) macrophages.

Received for publication 5 December 1977.

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EXPRESSION OF Ly 1, Ly 2, Thy 1, AND TL DIFFERENTIATION ANTIGENS ON MOUSE T-CELL TUMORS

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The "differentiation antigens", Ly 1 and Ly 2, present on thymus cells and thymus-derived lymphocytes (T cells)[1] of mice (1) have been shown to mark differentiated functional subpopulations of normal peripheral T cells. By using cytotoxic elimination experiments, it was reported initially that in C57BL/6 (B6) mice, an Ly 1^+ Ly 2^- (Ly 1 ↑, Ly 2 ↓)[2] subpopulation of peripheral T cells primed with sheep erythrocytes had helper activity for an in vitro sensitized trinitrophenol-specific antibody response; while among T cells of B6 mice immunized with cells allogeneic to B6, only those bearing the Ly 2 phenotype had killer activity in vitro against a fibroblast target of the same allotype as the cells used for immunization (2). In an expanded study, the Ly $1^-2^+3^+$ (Ly 1 ↓, Ly 2,3 ↑) phenotype of cytotoxic effector cells was confirmed in B6 mice, which express the Ly 1.2, 2.2, 3.2 alleles. However, in B6/*Ly 1.1* congenic mice which express the Ly 1.1, 2.2, 3.2 alleles (i.e., the congenic strain of mice in which the Ly 1.2 allele has been replaced by the Ly 1.1 allele) Ly $1^+2^+3^+$ cells also were demonstrated to be cytotoxic effector cells (3). Moreover, T cells which are precursors of killer or helper cells apparently are differentiated for these Ly antigens before immunization (4), although the point at which this differentiation takes place has not been ascertained. Also under certain experimental conditions T cells, expressing the Ly 1^-2^+ phenotype, are required for immune suppression (5). These initial observations of functional subsets with specific restricted Ly phenotypes have been confirmed, and extended to encompass a variety of T-cell functions (6–19).

Tumors with differentiated T-cell phenotypes would be potentially valuable for studies of T-cell function. Most T lymphocytic tumors in the mouse however, are spontaneous and induced tumors of thymic origin. The thymus is thought to be chiefly a lymphocytopoietic tissue containing relatively immature cell types. The original report in which Ly 1 and Ly 2 antigens were described (1) indicated that lymphomas varied in the expression of Ly antigens, although it was not reported whether the tumors expressed both Ly specificities when positive. That report also noted that the expression of TL, another T-cell antigen (20), was not correlated with the presence or absence of Ly antigen. Several questions about the phenotypic expression of Ly on neoplastic cells are not resolved: (*a*) Are both Ly 1 and Ly 2 antigens expressed equally on all T-cell tumors, or are the T-cell tumors, like peripheral T cells, differentiated for Ly phenotype?; (*b*) Are Ly antigens expressed only on T-cell lymphomas?, and (*c*) What is the relation of the antigenic phenotype to cell differentiation and leukemogenesis? To study these questions, we have examined early transplantation generations of a number of BALB/c lymphoid tumors induced by 1-ethyl-1-nitrosourea as well as early transplantation generations of spontaneous AKR lymphomas, for Ly 1, Ly 2, TL, and Thy 1 phenotypes.

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* Supported by the Cancer Research Institute, New York.

[1] *Abbreviations used in this paper*: ABS, absorption; B6, C57BL/6; C, complement; CT, cytotoxicity; ENU, 1-ethyl-1 nitrosourea; Fl-GAMIg or (heavy chain), fluorescent goat anti-mouse Ig or (Ig heavy chain); IF, immunofluorescence; T cells, thymus-derived lymphocytes; TL, thymus leukemia antigen.

[2] The +/− superscript nomenclature is a simplification of the conclusion from the original papers (2, 3) on functional subsets, one of which (3), suggested the Ly 1 ↓ :Ly 2,3 ↑ (i. e., arrows) nomenclature to indicate that the functional populations were relatively poor or rich in the respective antigens.

Materials and Methods

Mice. Mice used in these experiments for tumor passage were either BALB/c AnN, bred at the Animal Production Unit, National Institutes of Health, Bethesda, Md. or by Litton Bionetics, Kensington, Md., or AKR/J from The Jackson Laboratory, Bar Harbor, Maine.

Mice congenic with B6 were used for specificity controls with Ly and TL antisera, and were either obtained directly from Dr. E. A. Boyse, Memorial Sloan Kettering Cancer Center, New York, or bred in this Laboratory from stock obtained from Dr. Boyse. These congenic stocks are designated B6/*Ly 1.1*, B6/*Ly 2.1*, *Ly 3.1*, and B6/TL^+ following the simplified nomenclature of Shiku et al. (3). A/*Thy 1.1* mice congenic with A strain mice, but expressing the Thy 1.1 rather than the Thy 1.2 antigen, were obtained from Dr. David Sachs, National Cancer Institute, for specificity controls with Thy 1 antisera.

Tumor Induction and Transplantation. Specific tumor designations with further information for BALB/c tumors are shown in Table I. P1798, a long-term passage tumor line arose in a BALB/c mouse treated with estrogen (21). All of the other BALB/c tumors presented here are of recent origin and most were tested at less than 10 transplantation generations. These tumors were induced by 2 intraperitoneal injections of 1-ethyl-1-nitrosourea (ENU), 1 wk apart in 1- to 2-mo old animals. Each injection contained 1 μmol ENU/g body weight. The ENU was prepared and injected by Dr. Jerry Rice, National Cancer Institute. Some of the tumors arose in ENU-treated mice that were subsequently given a single i.p. injection of pristane (2, 6, 10, 14 tetramethyl pentadecane) 1 mo later (22). Eight of the BALB/c tumors presented as primary thymic tumors with or without evidence of generalized spread of the leukemic process while two of them BALENTL 13 and 14 arose as generalized leukemias. The BALB/c tumors were transplanted by serial i.p. or s.c. passage. Solid tumor tissue was minced into suspension, or peritoneal tumor ascites cells were used when available for the tests performed.

The AKR transplantable tumors, AKRLS-12, AKRLS-13, and AKRLS-34, were initiated by s.c. or i.p. transplantation of primary spontaneous thymic tumors of AKR/J mice > 4 mo of age. Pathology of these tumors was judged only on a gross level and was comparable to the frequently described pattern of lymphoid tumors for this strain of mice: grossly enlarged thymus; enlarged lymph nodes; and enlarged spleen in some cases. The only selective basis for inclusion in this study was the availability of early transplantation generations (generations 5–11). All of the tumors have been frozen in liquid N_2. Six recovered lines were tested including BALENTL 13, 14, P1798, and the 3 AKR tumors.

Antisera. Anti-Ly, -TL, and -Thy 1 antisera which were similar to those previously described (3), were a generous gift of Doctors E. A. Boyse and F-W. Shen, Memorial Sloan-Kettering Cancer Center, and are listed below (Table II). The antisera were selected for specificity of reaction only on thymocytes of B6 or the appropriate congenic strain. When necessary these antisera were absorbed with normal thymus and peripheral lymphoid tissue or tumors of the opposite allelic Ly specificity. The titers and dilutions of the antisera also are indicated in Table II.

Cytotoxicity Test. The cytotoxicity (CT) test of Gorer and O'Gorman (23) was used with the modifications described by Boyse et al. (24). Equal 0.05-ml vol of (*a*) antiserum serially diluted; (*b*) selected rabbit serum, diluted 1:15, in Ly tests (25, 26), or selected guinea pig serum, diluted 1:4, in TL and Thy 1 tests (both complement [C] sources were selected for low cytotoxicity against

TABLE I

*BALB/c Tumors**

Name	Induction	Days‡	Pathology		Transplantation generations tested¶
			Gross§	Microscopic‖	
BALENTL 3	ENU	132	T	S-LN, Thymus	3, 4
BALENTL 4	"	165	T	–	3¶
BALENTL 5	"	173	T	L-LN, Viscera	2, 3, 6, 15, 17
BALENTL 6	"	273	T	S-LN, Thymus	1, 2
BALENTL 7	ENU + Pristane	273	T	–	1¶
BALENTL 8	" "	311	T	S-LN, Thymus	1,3
BALENTL 9	" "	344	G	S-LN, Thymus, early spleen	3¶
BALENTL 13	" "	145	G	S-LN, Lymph node; RCS-B, Liver	3, 4,¶ 5,¶ 12
BALENTL 14	ENU	443**	G	L-LN, Viscera	4,¶ 12
P1798	Estrogen pellet	521**	T	S-LN, Thymus	189, 193, 194
BALENLM 11	ENU	260	G	S-LN, Lymph node, spleen (non-thymic)	2, 3, 4, 7, 10
BALENLM 15	ENU + Pristane	227	–	Giant cell sarcoma	1¶
BALENLM 16	" "	173	–	Microscopic plasma cell tumor	1¶
BALENLM 17	" "	235	G	S-LN (Mastocytoma)‡‡	1,¶ 2

*All of these tumors were induced in BALB/c AnN mice.

‡Days after treatment with ENU.

§T, thymic, G, generalized lymphoma.

‖S-LN, small cell lymphocytic neoplasia; L-LN, large cell lymphocytic neoplasia; RCS-B, Dunn reticulum cell sarcoma type B.

¶A number of these tumors were tested from more than one animal at the same transplantation generation. The transplantation generation number indicates the number of times the tumor was transplanted prior to testing.

**Actual age of mouse in days.

‡‡This tumor initially appeared to be mixed for cell type, but grew out as a mastocytoma on transplantation.

TABLE II

*Antisera**

Specificity	Immunization	Test Thymocytes	Maximal CT	Titer
Anti-Ly 1.1	(BALB/c × B6)F_1 vs. B6/*Ly 1.1* thymocytes	B6/*Ly 1.1*	>95%, 1/160	2,000
Anti-Ly 1.2	C3H/An vs. CE thymocytes	B6, BALB/c, AKR	>95%, 1/80	320
Anti-Ly 2.1	B6/*H-2*^{k} vs. CE thymocytes	B6/*Ly 2.1, 3.1*, AKR	>90%, 1/80	640
Anti-Ly 2.2	(C3H/An × B6/*Ly 2.1*)F_1 vs. ERLD‡	B6, BALB/c	>90%, 1/80	320
Anti-TL	(B6 × A/TL)F_1 vs. ASL1	BALB/c	70%, 1/200	1,000
"	"	A, B6/TL$^+$	>95%, 1/400	6,400
Anti-Thy 1.1	(B6 × A)F_1 vs. A/*Thy 1.1* thymocytes	A/*Thy 1.1*, AKR	>95%, 1/80	2,000
Anti-Thy 1.2	(A/*Thy 1.1* × AKR/*H-2*^{b})F_1 vs. ASL1‡	A, BALB/c	>95%, 1/500	5,000

*The maximal cytotoxicity is given as the percent dead cells at the serum dilution indicated. The titer is the reciprocal of the dilution which gives 50% lysis on the cells indicated.

‡ERLD and ASL1 are B6 and A strain tumors, respectively, which have long transplantation histories.

mouse thymocytes and high C activity); and (*c*) cells suspended in Medium 199 with Hanks' balanced salt solution (5 × 10^6 cells/ml) were incubated for 45 min at 37°C. The data are presented as percent dead (stained) cells after the addition of trypan blue in 0.15 M saline.

Immunofluorescent Reagents. Goat anti-mouse Ig heavy chain and anti-mouse polyvalent Ig antibodies were prepared by affinity chromatography and tested as described elsewhere (27).

Goat antibodies were coupled to fluorescein by reaction with fluorescein isothiocyanate to achieve a molar fluorescein: protein ratio of 2.5–5.7. Unconjugated fluorescein was removed by passage over Sephadex G-25 equilibrated with 0.01 M phosphate buffer in 0.15 M NaCl, pH 7.4. These fluorescent reagents are designated Fl-GAM (heavy chain) and Fl-GAMIg. All of these reagents react with <1% of normal thymocytes.

Immunofluorescence Test. The immunofluorescence (IF) test of Möller (28) was adapted as follows for use with mouse alloantisera. Equal vol (0.05 ml) of diluted alloantisera and cells prepared in Hanks' medium with 10% heat inactivated fetal calf serum and 0.1% sodium azide (20 × 10^6 cells/ml) were mixed on ice for 30 min. The suspensions were then diluted to 2 ml and washed twice with centrifugation at 220*g*. Appropriate Fl-GAM (heavy chain) or Fl-GAMIg (0.05

ml), diluted to 0.5 mg/ml protein in phosphate-buffered saline was added to the pellet. The cells were suspended and incubated on ice again for 30 min and washed as before. The washed cell pellets were suspended in 1-drop vol and put on slides with cover slips and read under oil immersion with a Leitz fluorescent scope with Ploem illuminator (HBO 100W/2 mercury lamp with BG38 and BG12 excitation filters and an S546 barrier filter).

The use of the Fl-GAMγ_2 reagent allowed us to type Ig$^+$, H chain$^-$ tumors for Ly phenotype as discussed below. (See Results)

Absorption Tests. Tumors or normal lymphoid tissues for controls were prepared by suspension in Medium 199 with Hanks' balanced salt solution. The cells were washed twice, resuspended in 3–5-ml vol, and enough cells to yield a 0.05-ml cell pellet were dispersed into 50 × 6-mm tubes. After centrifugation at 800 *g* the supernate was aspirated and a 0.11-ml of appropriately diluted antiserum was added and mixed with the cells. The dilution of antiserum used in these tests was determined previously to be a dilution at which maximum positive reaction (either 90–95% CT or >90% IF) was observed on the appropriate thymus test cels. The absorptions (ABS) were carried out by mixing the cells on ice for 45 min, centrifuging the absorbing cells into a pellet, and transferring the absorbed serum supernate to a fresh tube for either a standard CT assay with serial twofold dilutions or IF on an antigen-positive thymus cell. This is the most critical test because antiserum specificity can be completely controlled by the use of B6 or congenic mouse thymocytes as the positive test cell after the absorption. Positive (+) ABS is indicated when no reactivity above complement controls or <1% IF was observed on the positive test cells. Negative (−) ABS indicates that no reduction was observed greater than that obtained with negative control, lymphoid tissue of mice with the opposite Ly (Thy 1 or TL) allele. Usually this does not exceed 5–10%. Exceptions are noted in the Tables.

Results

Preferential Expression of Either Ly 1 or Ly 2 on Lymphoma Cells. Table III presents a summary of the CT, IF, and absorption data for the BALB/c and AKR tumors. 11 of these tumors were tested by all three tests. All of the tumors were tested at least twice and some of the tumors were tested a number of times (see Table I and below).

CT Tests on BALB/c Tumors. Typical CT data for two of the tumors tested with anti-Ly 1.2 and anti-Ly 2.2 are present in Fig. 1. A high level of CT >90% at the 1/80 dilution of anti-Ly 1.2 was observed for BALENTL 13, while CT for BALENTL 5 was 28% under the same conditions. CT with anti-Ly 2.2 indicated that BALENTL 5 cells were >90% positive (BALENTL 13 CT under the same conditions was <10%). The initial dilutions of both antisera were chosen to be on a maximum plateau of kill for normal BALB/c thymocytes. In Table III the CT data for these tumors and other similar tumors are presented in summary at this plateau dilution. It is evident that some of these tumors had high levels of expression of either Ly 1 or Ly 2 antigen, indicated as Ly 1$^+$ or Ly 2$^+$, comparable to the Ly phenotypic expression of normal thymus cells, but clearly both antigens were not expressed to the same degree. With these criteria BALENTL 3–8 and 14 are Ly 1$^-$2$^+$ and BALENTL 13 is Ly 1$^+$2$^-$. It also is evident that two of the BALB/c lines, P 1798 and BALENTL 9 were positive for both Ly antigens. The level of CT, however, was lower for both of these tumors compared to normal thymocytes.

CT results on tumor cells prepared from lymphoid or solid tumor tissues suggested that some of the tumors which were scored as negative for one of the Ly antigens had a subpopulation of about 15% positive cells. This low degree of positive reactivity was absent in ascites tumors.

Occasionally these tumors were tested with antisera to Ly 1.1 or Ly 2.1, the

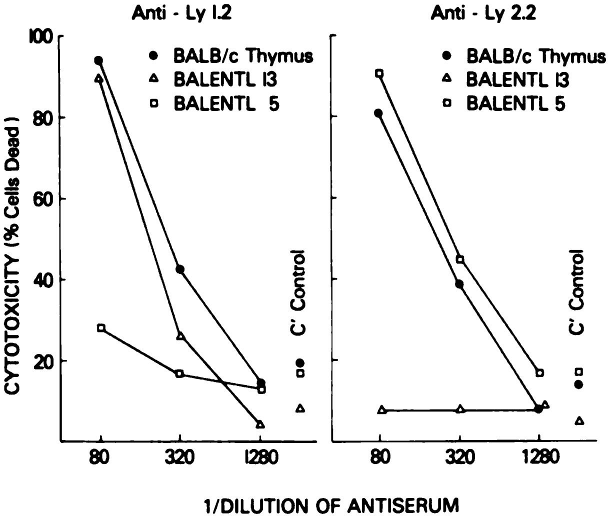

FIG. 1. Cytotoxicity of anti-Ly 1.2 and anti-Ly 2.2 on BALENTL 13 and BALENTL 5. Cytotoxicity = percent dead cells (stained with trypan blue) on ordinate; 1/dilution of antiserum on abscissa. BALENTL 13 was tested at generation 5 in ascites form (See Table VI) and BALENTL 5 was tested at generation 2 as a suspension of a solid subcutaneous tumor. Control normal BALB/c thymus cells were obtained from a 2-mo old animal. Serum controls (serum 1:80, without C added, not pictured) were <10% for both antisera with either tumor.

opposite alleles to those detected on BALB/c cells, and no positive tumors were observed with either antiserum.

BALENLM 11, 15, and 17 tumor cell suspensions had reduced phenotypic expression of both Ly 1 and Ly 2 antigens and were also Thy 1.2^- and TL^-.

IF Assay of Ly Antigen Expression. Because CT tests as performed here with anti-Ly sera cannot distinguish with satisfaction reactions on subpopulations of cells, we developed immunofluorescence tests for use with mouse alloantisera. This approach had the potential of asking whether tumors with low antigen expression by CT, e.g., 20% had (*a*) reduced antigen expression on all the tumor cells thus reducing the sensitivity of the cells to lysis with antibody and C, or (*b*) a minor subpopulation of positive cells with normal thymus levels of Ly antigen expression.

With these high CT anti-Ly sera, either Fl-GAMIg or Fl-GAMγ_2 reagent was satisfactory for IF tests on normal thymus cells, because the maximum number of Ly^+ cells were labeled with either reagent. However, the fluorescence intensity of the Fl-GAMγ_2 was lower and therefore the Fl-GAMIg reagent was employed whenever possible. When tested, Fl-GAMγ_1, Fl-GAMIgA, and Fl-GAMIgM did not give satisfactory stains with these anti-Ly sera. The specificity of the sera used in this report was confirmed by tests on the appropriate congenic thymocytes. Under the conditions described, fluorescent dots on >90% of the cells could be seen when the sera had been selected and/or absorbed to react specifically with thymocytes from B6 or the proper Ly congenic strain. Since the Fl-GAMγ_2 reagent reacts with <1% of spleen cells and <5% of Ig^+ tumor cells, this reagent, when used with anti-Ly sera, allowed us to test Ig^+

TABLE III

*Antigen Distribution on BALB/c and AKR Tumors**

Tumor	Ly 1.2			Ly 2‡			TL			Thy 1‡	
	CT	IF	ABS	CT	IF	ABS	CT	IF	ABS	CT	ABS
BALENTL 3		<5			95			>90		95	
BALENTL 4	20			90			95				
BALENTL 5§	21	11	–	>90	>90	+	>95	72	+	>95	+
BALENTL 6	14	<5	–	52	>95	+	>95	95	+		+
BALENTL 7	15		–	95		+			+		(60%)‖
BALENTL 8	<10		–	36	>95	+	>90		+	51	+
BALENTL 9	31	>95	+	50	>95	+	76	>95	+	76	+
BALENTL 13§	90	90	+	10	<5	–	<10	<10	–	34	+
BALENTL 14		<1–20	–		58–90	+	>90	>90	+	>95	+
P 1798	50	50	+	27	90	+	>95	>95	+	43	+
BALENLM 11¶	(–)	27		(–)	22		(–)	1		16	+
BALENLM 15	28	<5		28	<5		<5			<10	
BALENLM 16¶		<5			13			<5			
BALENLM 17	24	11	–	4	<5	–	<10	<1	–	<5	
AKR LS 12	>90			<10			<5				
AKR LS 13	<5	<5	–	51	90	+	<5	<1	–	23	+
AKR LS 34	21	<1	–**	42	95	+	21	<5	–	17	+

*CT, percent dead (trypan blue stained) cells. CT data are presented at the 1/80 dilution for anti-Ly 1.2, 1/40 or 1/80 for anti-Ly 2.2, 1/200 for anti-TL, 1/500 for anti-Thy 1.2, 1/40 for anti-Ly 2.1 and 1/80 for anti-Thy 1.1. IF, percent fluorescent cells. Antisera to Ly 1, Ly 2, and TL were routinely used one twofold dilution less than indicated for CT tests. Control cell preparations incubated with Fl-GAMIg were routinely <5% except for BALENLM 11, 15, 16, and 17, which were 37->95% Ig^{+} but which were <1% positive with Fl-GAM$_{\gamma 2}$. ABS (absorption data) are summarised as: positive ABS (+), <1% IF (or <5% CT) on positive indicator cells. Negative ABS (–), 90–95% IF or CT on positive indicator cells. B6 or B6/Ly congenic thymocytes were used for testing absorbed anti-Ly sera, BALB/c thymus cells were used for testing absorbed anti-TL, and A/J or A/Thy 1.1 thymus cells were used for testing absorbed anti-Thy 1. C controls on tumors were about 10% (5–10) in most cases.

‡The Ly 2.2 and Thy 1.2 alleles are expressed on normal BALB/c thymocytes and BALENTL lymphomas. The Ly 2.1 and Thy 1.1 alleles are expressed on AKRLS lymphomas and normal AKR thymocytes.

§More detailed data are presented for BALENTL 5 and BALENTL 13 in Tables IV and V.

‖This tumor absorbed 60% of the anti-Thy 1.2 CT compared to complete absorption with an equal volume of normal packed BALB/c thymus cells.

¶CT results were not greater than controls 15–20% dead cells, however, cell loss during the test incubation was a major problem with BALENLM 11 as well as for BALENLM 16.

**Under the conditions described, partial absorption of 30% was observed. This low degree of absorption was assumed to be from contaminating host cells (see text).

tumors for Ly antigen. For tests of Ig^{+} tumors, the Ly sera were centrifuged at 100,000 *g* for 20 min in a Beckman Airfuge (Beckman Instruments, Fullerton, Calif.) to remove immunoglobulin complexes and aggregates.

When lymphoid tumors (BALENTL 3, 5, 6, 8, 13, and 14) were tested by IF for Ly expression and when only large or intermediate sized cells (i.e., presumptive tumor cells) were scored for fluorescence, the tumor cells consistently appeared to be totally negative for one of the Ly antigens and strongly positive for the other. The pattern of surface dots with anti-Ly sera and Fl-GAMIg was quite distinct and was similar on normal thymus and tumor cells. The question of low positive reactions was clarified by IF tests since a subpopulation of smaller cells was observed with brightly fluorescent dots, probably indicating a contaminating host cell population rather than a low level of antigen expression on the total population of tumor cells.

Lack of Ly on Ig^{+} Tumors. Initial attempts to type several of the BALB/c tumors by cytotoxicity tests were unsatisfactory because of a depletion of test cells during the 37°C incubation with C. When BALENLM 11, 15, 16, 17, were tested for surface Ig by IF with polyvalent Fl-GAMIg, a clear pattern of fluorescent membrane dots and rims was observed. This suggested that these tumors either had bound Ig passively in vitro or had intrinsic surface Ig. These surface Ig^+ tumors were subsequently tested for Ly and TL antigens by IF with Fl-GAMγ_2. In these tests, where loss of tumor cell was not a problem, the Fl-GAMγ_2-negative tumors were also negative with either anti-Ly 1.2 or anti-Ly 2.2 sera. Preliminary tests of ABLS 5, PL 1, and PL 2, Abelson virus-induced tumors, also indicate a lack of Ly antigens on these tumors (M. Potter and B. J. Mathieson, unpublished data).

Expression of TL and Thy 1.2 Antigens on BALB/c Tumors. Two of the tumors, P 1798 and BALENTL 9, undifferentiated for the T-cell markers Ly 1 and Ly 2, similar to normal thymus cells, are TL^+. In addition most of the BALB/c tumors with a single Ly phenotype also express TL. On at least two of the Ly 2^+ TL^+ tumors, BALENTL 5 and 6, the TL specificities observed are at least TL.2 and TL.1, 4 or 5 (20, 29); and TL.3 is not expressed in addition to the normal TL.2 specificity. This conclusion is drawn from the following observations: anti-TL serum absorbed with either BALENTL 5 or 6 remains cytotoxic on either A strain or B6/TL^+ thymocytes but not on BALB/c thymocytes, and anti-TL serum absorbed with ERLD, a TL.1, 2, 4 positive tumor, removes cytotoxic reactivity to the BALENTL tumors.

Most of the tumors were tested for Thy 1. CT with anti-Thy 1 sera is often greatly reduced on tumors but maximal CT of 95% could be obtained with increased serum concentrations. Only the T-cell tumors tested, i.e., tumors that were Thy 1^+ and/or TL^+, express Ly antigens, while several non-T-cell tumors do not.

Absorption Tests for T-Cell Antigens. Since CT and IF tests are direct tests on tumors, it was necessary to confirm the tumor cell phenotypes by absorbing known positive sera with tumor cells under experimental conditions which would allow maximum sensitivity, and then testing the absorbed sera on positive indicator normal thymocytes to check for specific ABS. The summary table (Table III) indicates those tumors which were tested by ABS and the results completely agree with the phenotypes determined by CT and IF. On some of the tumors, the phenotype was confirmed for all of the T-cell antigens by ABS tests.

Stability of Surface Phenotype in BALB/c T-Cell Lymphomas. The surface phenotype is stable over a number of generations, as demonstrated by BALENTL 13, an Ly 1.2^+, 2.2^- tumor (Table IV) and BALENTL 5, an Ly 1.2^-, 2.2^+ tumor (Table V). Repeated tests on BALENTL 13 indicated an Ly 1^+2^- phenotype in two sublines and on different tumor cell sources within a single transplant host. The isolated observation of a substantial Ly 2^+ population in a solid peritoneal tumor mass was not repeated when tested several generations later and remains unexplained.

AKR Tumors. Transplanted AKR Thy 1.1^+ lymphomas (AKRLS 12, 13, 34) also appear to have restricted expression of Ly antigens (Table III). Preliminary data (not shown) from an additional group of AKR thymic lymphomas have indicated that the one notable difference between these tumors and BALB/c tumors is the low incidence of TL^+ AKR tumors, which is consistent with previously reported data for lymphomas of this strain (20). Nearly all (9 of 10) of the BALB/c T cell tumors and 0 of 3 of the AKR tumors presented here are TL^+.

TABLE IV

*Ly Antigen Phenotype of BALENTL 13 – Repeated Testing**

Transplantation generation and subline‡	Tumor cell source	Ly 1.2			Ly 2.2			TL		Thy 1.2	
		CT	IF	ABS	CT	IF	ABS	CT	ABS	CT	ABS
3-S	Ascites	>95 (C 16)			15 (C 12)						
	Mesenteric lymph node	>95 (C <10)			25 (C <10)						
4-A	Ascites	85 (C <10)			<5 (C <5)			<10 (C <10)			
	Solid peritoneal tumor mass	78 (C <15)			51 (C <15)			31 (C <17)			
4-S	Ascites		>95 (>1)	+		4§ (<1)	-				
5-S	Ascites	90 (C <5)			10 (C <5)			12 (C <10)		34 (C <5)	
12-S	Solid peritoneal tumor mass		>90	+		<5	-		-		+/<10‖
17-A	Ascites		>95			<1				18 (C <5)	+/57‖

*Sera were used as described in Table III and tests were performed as detailed in the text. C controls are indicated in parentheses below the test results. This tumor was consistently Ig (<5%) when tested by IF at generations 4, 12, and 17.

‡The number indicates the passage generation; the letters S and A indicate whether the line was initially passed from the primary solid tumor in the chest cavity (S), or from the peritoneal ascites cells of the primary tumor (A).

§No large tumor cells positive.

‖AKR tumors (Thy 1.1^+) used for control absorptions did not decrease the CT on A/J thymus cells in either case.

TABLE V

*Ly Antigen Phenotype BALENTL 5 – Repeated Testing**

Transplantation generation	Tumor cell source	Anti-Ly 1.2			Anti-Ly 2.2			Anti-TL				Anti-Thy 1.2	
		CT	IF	ABS IF on B6	CT	IF	ABS IF on B6	CT	IF	ABS CT on BALB	ABS CT on A	CT	ABS CT on A
2	SC	28			>90			>95					
3	SC	21	11		>90	>95		>95	80				
6	SC		15	–/>90		80	+/<5		64	+/<15	–/>90	>90	+/<5
15	ASC	15	(+)§		39	>95		>95	>95			76	
15	SC		(+)§			>95		>95	>95			>95	
17	ASC	22	<10	–/>90	50	>95	+/<5	>95	>95	+/<15	–/82	54	+/<5
17	IP Solid	20	<10		37	>95		>95	>95			85	

*Sera were similar to those described in Table II and tests were performed as described in the text. This tumor was consistently Ig^- (<5%) each time it was tested by IF.

‡Most of these positive cells were smaller than the typical tumor cell.

§Although these cells appeared slightly positive the typical dot pattern of IF with anti-Ly was not observed with these cells. Since this tumor appears to have some viral-related specificities, this positive result may be an artifact. A subsequent generation tested by absorption was negative for Ly 1.2.

Discussion

We have used three methods for testing the Ly and TL phenotype of T-cell tumors of BALB/c and AKR mice and two methods for detecting Thy 1 antigen. In 11 of the 13 tumors either Ly 1 or Ly 2 antigen is expressed at a level comparable to Ly antigen expression in the thymus and the tumors are "negative" for the other Ly antigen. Absorption and C-dependent cytotoxicity have indicated no more than a minor background (probably normal host cell contamination) of positive cells for one of the Ly antigens: IF has indicated that large tumor cells are negative for the corresponding antigen. However, we cannot eliminate the possibility that the phenotypic expression is below the level of sensitivity of our tests, for the "negative" Ly antigen. Nor can we eliminate the possibility that the minor population of small Ly 1^+2^+ cells present in the in vivo passaged tumors are stem cells for large tumor cells with restricted Ly phenotype. This is unlikely in view of preliminary observations which have indicated a lack of small positive cells among cells of established in vitro lines of these tumors.[3] Therefore we conclude that there is preferential but not necessarily exclusive expression of either Ly 1 or Ly 2 on most tumors of these strains.

It is remarkable that so many of the thymic tumors described here are "differentiated" for Ly 1 or Ly 2 since nearly all thymus cells express both antigens. Partial lysis and sequential lysis experiments (2, 4) have distinguished four subclasses of normal peripheral T cells with phenotypes indicated as follows: Ly 1^+2^+; Ly 1^+2^-; Ly 1^-2^+; Ly 1^-2^-. Thus these tumors may reflect (*a*) neoplastic induction of a partially differentiated thymus cell or alternatively, (*b*) neoplastic conversion of a peripheral T cell which returns to the thymus to produce a tumor. The first possibility is more likely since the majority of the BALB tumors are TL^+.

TL antigen is expressed on thymus cells and on tumors of presumed T-cell origin but has not been found on peripheral T cells (20). Since some tumors are Ly 1^-2^-[4] or Ly 1^+2^- and also TL^+, the presence of TL on these tumors might represent the derepression of a tumor-specific TL antigen after differentiation has restricted the surface phenotype to either Ly 1^+ or Ly 2^+. Although the BALB/c tumors have not been tested for all of the TL specificities, it appears that most of these tumors express more than the normal BALB/c thymus TL.2 specificity. However, if the TL^+ phenotype is simply the result of derepression of tumor-specific TL, it is peculiar that the majority of these tumors (9 of 10) derived from a $TL.2^+$ strain (BALB/c) are TL^+ tumors while the majority of tumors in AKR, a TL^- strain are TL^- (20, 30). Thus the proportion of TL^+ tumors reflects the TL genotype of the target for leukemogenesis.

The normal differentiation process may involve the restriction of Ly before the differentiative loss of TL. The tumor phenotype might indicate that the target cell for leukemogenesis is a partially differentiated thymocyte. In spontaneous leukemogenesis of AKR mice (31, 32), radiation leukemogenesis and RadLV virus-induced leukemogenesis in B6 mice (31) the preleukemic thymus becomes populated with cells expressing a low Thy 1, high H-2 antigen phenotype. Such a phenotype is characteristic of peripheral T cells rather than thymocytes which normally are high Thy 1, low H-2 cells. The tumors presented here were also characteristically less susceptible to lysis with anti-Thy 1 and C. Thus the target cell population for thymic leukemogenesis may represent a normally minor thymus cell population which has reduced Thy 1 expression quantitatively and at least in part is differentiated for Ly antigen expression. Since a subpopulation of thymocytes, capable of synergy in graft-versus-host reactions, are eliminated with anti-TL serum (33), there may indeed be a normal phenotypic counterpart to the type of cell observed in these tumors. Thus, we may have to re-examine the model of normal T-cell differentiation (16, 34) which proposes that normal thymocytes lose the potential for TL expression before the differentiation of subpopulations restricted for Ly 1 or Ly 2, and before the acquisition of the capacity to react specifically with antigens. In addition the following model of T-cell differentiation should be considered:

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[3] Kim et al. Manuscript in preparation.

[4] Preliminary examination of 3 (BALB/c × DBA/2)F_1 tumor transplantation lines suggests that one of these lines is Ly 1^-2^- and TL^+.

$$\begin{array}{llll} & \mathrm{TL}^+\ \mathrm{Ly}\ 123 & \rightarrow & \mathrm{Ly}\ 123 \\ \mathrm{TL}^+\ \mathrm{Ly}\ 123 \rightarrow & \mathrm{TL}^+\ \mathrm{Ly}\ 1 & \rightarrow & \mathrm{Ly}\ 1 \\ & \mathrm{TL}^+\ \mathrm{Ly}\ 23 & \rightarrow & \mathrm{Ly}\ 23. \end{array}$$

An alternative explanation for the high proportion of TL$^+$ tumors exists: the anti-TL serum used in these tests may detect Qa-2 or T-cell differentiation antigens related to TL by genetic proximity on chromosome 17 (35). This question is still under investigation.

The stability of the phenotype over several generations and the finding of tumors with either the Ly 1$^+$ or Ly 2$^+$ phenotype supports the concept that these tumors represent states of T-cell differentiation. The stability further indicates that these antigens are not subject to modulation during transplantation of these tumor lines, but rather that the restricted Ly surface phenotype is an intrinsic property of these T-cell tumors.

Although there appears to be a predominance of BALB/c lymphomas with the Ly 1$^-$2$^+$ phenotype, this is not true of AKR lymphomas. The Ly phenotype may depend on the method of tumor induction, the strain of mice used, or it may simply reflect a sampling error due to the relatively small number of tumors tested from these strains. Eight of the BALB/c primary T-cell tumors presented as thymic tumors with or without evidence of generalized spread of the leukemic process, while three tumors, each with a different Ly phenotype, BALENTL 9, 13, and 14, arose as generalized T-cell leukemias possibly originating from peripheralized T lymphocytes.

Tumors have been described which subserve functions such as suppression (36), or autoaggression (37, 38), the Ly phenotype of those tumors may correspond with previously described phenotypes of normal functional immune cells. Since there is no exclusion of either of the restricted Ly phenotypes in these tumors, we find no support for the concept that all T-cell lymphomas will express the Ly phenotypes described for normal killer and suppressor or normal helper cells. However, individual tumor lines with such phenotypes may express appropriate functions under particular experimental conditions.

Beside the advantage of obtaining homogeneous populations with functional activity, these tumors have other obvious uses. In general, tumors with restricted Ly phenotypes may be useful as immunogens. BALENTL 13 has been used to produce anti-Ly 1.2 serum (F-W. Shen, personal communication). Since tumor tissue can be obtained in large amounts, and with the apparent restriction of the Ly antigen these tumors will be invaluable as a source for biochemical preparation of the antigen. Finally, the satisfactory description and availability of tumors such as these as models for T cells may enable us to expand our knowledge of the cellular aspects of the immune response as much as plasmacytomas have enabled investigators to expand our knowledge of B cells and antibody in immune responses.

Summary

Transplanted lymphomas, most of thymic origin, induced in BALB/c mice with 1-ethyl-1-nitrosourea (ENU) and transplanted spontaneously occurring lymphomas of AKR mice were examined for the expression of the T-cell antigens Ly, TL, and Thy 1 by using three serological methods. Most (11 of 13) of the Thy 1$^+$ and/or TL$^+$ tumors, i.e., T-cell tumors, expressed high levels of either Ly 1 or Ly 2 antigen, but not both. Thus most thymic lymphocytic tumors expressed restricted Ly phenotypes comparable to phenotypes previously described for functional peripheral T cells. Because tumor phenotypes were stable over a number of transplant generations, they therefore appeared to be an intrinsic property of the specific tumors. The majority of the BALB/c lymphomas were Ly 1$^-$ 2$^+$ and also positive with anti-TL antiserum. This predominant phenotype on the BALB/c tumors may be related to either the mode of tumor induction or to the mouse strain, but since the restricted Ly pattern was observed both in BALB/c and AKR tumors, the phenotypic restriction itself is not a consequence of either of these factors. Tumor induction by ENU per se is not responsible for Ly or TL antigen expression since several non-T-cell BALB/c tumors, also induced by ENU, did not express either Ly or TL antigens.

Data presented here suggest that the target cell for leukemogenesis may be a partially differentiated thymus cell. The restricted expression of Ly antigens on differentiating thymus cells to either the Ly 1$^+$ (Ly 1$^+$ 2$^-$) or Ly 2$^+$ (Ly 2$^+$ 1$^-$) phenotype may occur before the loss of TL antigen.

The authors gratefully acknowledge the technical assistance of Judy Wax and Janet Pumphrey and the extensive help and patience of Olive Childers in preparing the manuscript. We are indebted to Doctors E. A. Boyse and F.-W. Shen for their advice and generous supply of Ly antiserum and we wish to thank Doctors F. Weinbaum and H. C. Morse, III for their critical review of the manuscript.

Received for publication 18 April 1977.

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Brief Definitive Report

RELATIONSHIPS OF gp70 OF MuLV ENVELOPES TO gp70 COMPONENTS OF MOUSE LYMPHOCYTE PLASMA MEMBRANES*

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The term gp70 has been applied to the major envelope component of murine leukemia virus (MuLV), primarily on grounds of chemical characteristics and mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (1). However, antisera to MuLV or MuLV constituents react with certain cells that are not producing virus, yielding plasma-membrane molecules that are not distinguishable from viral gp70 by such criteria and so are also called 'gp70' (2–6). These gp70 species that compose part of the cellular plasma membrane may show mendelian inheritance (7) and genetic programming in the normal course of differentiation (8).

Three variants of the plasma-membrane gp70 class of molecules, – G_{IX}-gp70, X-gp70, and O-gp70 – have been categorized by immunogenetic methods, and Table I shows the cell-surface gp70 phenotypes of thymocytes from several mouse strains. We have now made peptide maps of tryptic digests of gp70 bands obtained by PAGE of immunoprecipitated lymphoid cell lysates and compared these maps with maps of plasma-membrane gp70s from cultured cells infected by ecotropic or xenotropic MuLVs.

We used MuLVs from our own cloned stocks (9). AKR ecotropic virus 69E5 and AKR xenotropic virus 69X9 were isolated from the thymus of the same 6-mo-old AKR mouse. [^{3}H]glucosamine-labeled viruses were prepared according to Tung et al. (4).

Cultured cells were grown in Dulbecco's modified Eagle minimal essential medium supplemented with 10% fetal calf serum. Productively infected cell cultures were washed, dispersed with EDTA, and harvested by centrifugation (9). Thymocytes were obtained from 2-mo-old female mice of our colonies. RADA1 and ASL1 are the transplanted A strain leukemias we studied earlier (5).

Cells were surface-iodinated with ^{125}I by the lactoperoxidase method and lysed with Nonidet P-40 (4). The lysates were reacted with antisera, and the antigen-antibody complexes precipitated with α-Ig. The immunoprecipitates were analyzed by SDS-PAGE on a Laemmli-type 5–17% gradient slab gel (10). The gp70 bands located by autoradiography were excised, and SDS removed by washing extensively with 50% methanol and 10% acetic acid followed by 10% methanol. The gel slices were dried by infra-red heat and subjected to tryptic peptide mapping (11). In brief, the gel slices containing gp70 were digested with trypsin (TPCK) (25 μg in 1 ml of 1% NH_4HCO_3). The digests were lyophilized to dryness, dissolved in buffer I (acetic acid:formic acid:water, 15:5:80), spotted on to 10 cm × 10 cm cellulose-coated thin layer plates, and electrophoresed at 900

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* Supported by grants CA-08748 and CA-16599 from the National Institutes of Health.

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J. EXP. MED. © The Rockefeller University Press · 0022-1007/78/0401-1280$1.00

TABLE I

Cell-Surface gp70 Phenotypes of Mouse Thymocytes

Antigenic sub-species of gp70	Mouse strains						Reference
	A	AKR	G_{IX} congenic strains				
			B6	B6-G_{IX}^{+}	129	129-G_{IX}^{-}	
G_{IX}-gp70	+	+	–	+	+	–	4,7
X-gp70	–	+	–	–	–	–	5
O-gp70[*]	ND[‡]	ND	+	+	–	–	6

[*] O-gp70 was originally identified on normal B6 thymocytes as a species of gp70 with the following properties: (*a*) precipitation by group-specific α-gp70 serum (*eg* α-R-MuLV gp70), (*b*) characteristic electrophoretic mobility in SDS-PAGE vis-à-vis G_{IX}-gp70 of 129 thymocytes, and (*c*) absence of type specificities of G_{IX}-gp70 [recognized by antibody in 'α-NTD' rat serum, (W/Fu × BN)F_1 α-W/Fu(C58NT)D (7), that is cytotoxic for G_{IX}^{+} thymocytes] and of X-gp70 [recognized by precipitating antibodies in α-X.1 serum, (BALB × B6)F_1 α-BALB radiation leukemia RL♂1 (5)]. In serological terms, O-gp70 is thus defined negatively, because no antisera identifying its type-specificity (or its type-specificities if it comprises more than one sub-species of gp70) have yet been discovered; hence the designation 'O-gp70'.

[‡] Not determined.

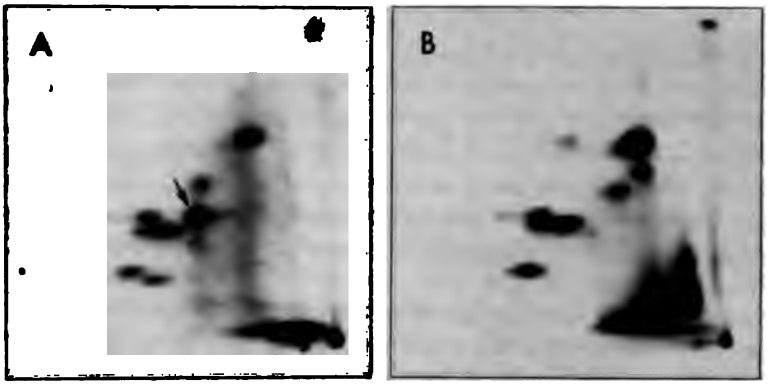

FIG. 1. Autoradiographs of ^{125}I-tyrosine-containing peptide maps of plasma-membrane gp70s of MuLV-infected tissue culture cells: (A) SC-1 mouse cells infected by AKR ecotropic virus 69E5, and (B) CCL64 mink cells infected by AKR xenotropic virus 69X9. Arrow indicates the 'A-spot'.

V for 20 min. The plates were dried, subjected to chromatography in buffer II (butanol:pyridine:acetic acid:water, 32.5:25:5:20), then dried and analyzed by autoradiography on Kodak XR-2 X-ray film with Cronex intensifying screens at −70°C.

Fig. 1 shows peptide maps from gp70s immunoprecipitated from MuLV-infected cultured cells. Goat α-R-MuLV gp70 antiserum was used for precipitation because of its broad group-specific reactivity. The two patterns shown, for (A) mouse fibroblasts infected with ecotropic AKR virus (AKR 69E5), and for (B) mink cells infected with xenotropic AKR virus (AKR 69X9), are readily distinguishable. The prominent 'A-spot' marked with an arrow on map A and missing from map B is a useful distinguishing feature. Maps from gp70s immunoprecipitated from virions gave patterns similar to those for infected cells. Thus virions of two ecotropic viruses (AKR-L1 CLG12 and BALB N-tropic WN1802N, reference 9) gave gp70 maps like pattern A (including the A-spot) whereas virions of two xenotropic viruses [AT124 and S16CL10(I), reference 9] gave gp70 maps like pattern B (no A-spot).

The two gp70 patterns in Fig. 1 (cultured cells infected with ecotropic or

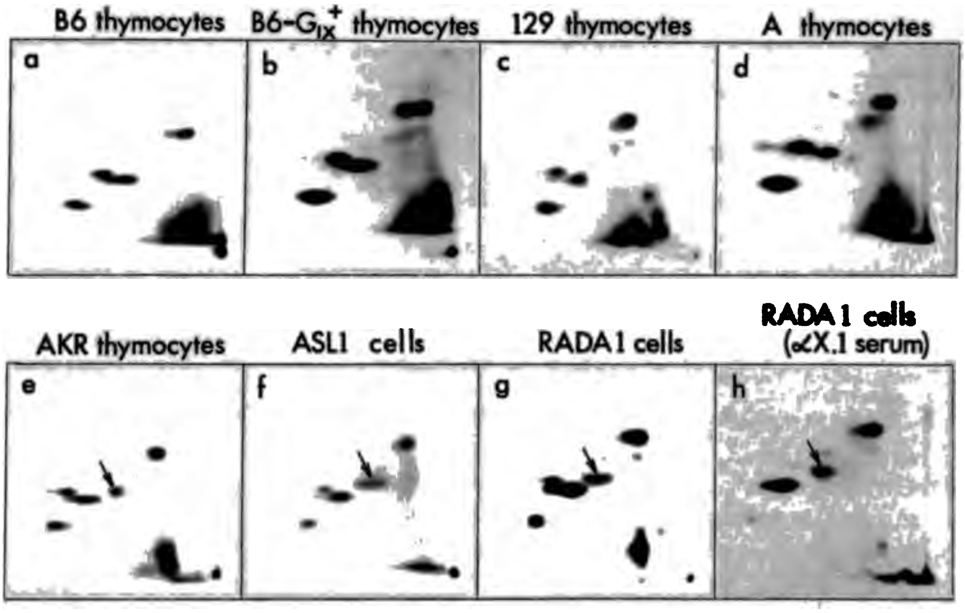

FIG. 2. Autoradiographs of ^{125}I-tyrosine-containing peptide maps of mouse cell plasma-membrane gp70s. Arrows indicate the A-spot.

xenotropic virus) were compared with maps of gp70 from seven mouse cell populations, comprising thymocytes of five mouse strains (Table I), and two A strain leukemias, RADA1 and ASL1, known to be X-gp70-positive (5). The seven maps are shown in Fig. 2.

(*a*) Maps *a* to *d*, for thymocytes of strains B6, B6-G_{IX}^+, 129 and A, show no pronounced differences. Thus G_{IX}-gp70 and O-gp70, though serologically distinct (Table I), are not distinguished by differences in peptide maps under the conditions used (which reveal only tyrosine-containing peptides).

(*b*) The same four maps (*a* to *d*), resemble map B of Fig. 1 (xenotropic virus gp70) more closely than map A (ecotropic virus gp70), notably in lacking the A-spot. Thus G_{IX}-gp70 and O-gp70 of thymocytes of these four strains seem related to xenotropic virus.

(*c*) Maps *e* to *g*, for AKR thymocytes and the two A strain leukemias, are broadly similar to one another and present a second pattern for gp70 of lymphocyte plasma membranes. This is more like A of Fig. 1 (ecotropic virus gp70), including the A-spot.

(*d*) Sequential precipitation (5) indicates that each of the leukemias expresses at least two gp70s. Thus gp70 patterns from these cells could be composite if these two gp70s give distinguishable maps. Map *h* shows this is probably so. For map *h* the lysate of RADA1 leukemia cells was precipitated with α-X.1 serum, which reacts with only one of the gp70s on this cell population (5). Some components of map *g* are missing from map *h*, and the missing elements therefore belong to a second gp70, other than X-gp70.

(*e*) Map *h* retains the A-spot which distinguishes ecotropic MuLV gp70 from xenotropic MuLV gp70 in the materials tested. The A-spot can therefore be assigned in this instance to X-gp70. The resemblance of map *h* to gp70 of ecotropic rather than xenotropic MuLV accords with the precipitation of gp70s

TABLE II

Discrimination of MuLV gp70 Types by α-X.1 Serum

^{3}H-Glucosamine-labeled MuLV (reference 9)	gp70 Precipitated by α-X.1 (% total gp70)[*]
Ecotropic:	
Gross Passage A	92[‡], 98, 100
AKR	92, 95, 101
WN1802N (BALB Endogenous)	96, 102
WN1802B (BALB Endogenous)	65, 70
B6N (B6 Endogenous)	82, 90, 93
B6B (B6 Endogenous)	89, 92
RL1 (From a BALB radiation-induced leukemia)	98, 102
Rauscher	7, 8, 10
Xenotropic:	
AT124 (From NIH/Swiss mice)	<1, <1, <1
S16 CL10(I) (From BALB/3T3 cell line)	<1, <2, <1

[*] cpm of gp70 PAGE gel band from α-X.1 precipitate ÷ cpm of the same band from α-R-MuLV gp70 precipitate × 100. The quantities of labeled virus used were equivalent, and the same dilution of each antiserum was used for all determinations with that antiserum.

[‡] Individual values represent separate experiments.

of several ecotropic but not xenotropic virions by α-X.1 serum (Table II).

There is further evidence that G$_{\text{IX}}$-gp70 is associated with xenotropic MuLV: mouse strain 129 and its congenic partner 129-G$_{\text{IX}}^{-}$ are genotypically similar except for the locus *Gv-1*. G$_{\text{IX}}$-gp70 is present on thymocytes and in the serum of 129 but not 129-G$_{\overline{\text{IX}}}$ mice (12, 13). Thus *Gv-1* controls expression of G$_{\text{IX}}$-gp70 in both situations. Map *c* of Fig. 2 represents the G$_{\text{IX}}$-gp70 molecule recognized in the 129 thymocyte plasma membrane by the G$_{\text{IX}}$ cytotoxicity assay. Also Elder et al. (11) report that the tryptic peptide map of gp70 found in 129 but not 129-G$_{\overline{\text{IX}}}$ serum resembles that of xenotropic virus from NZB mice. And radioimmunoassays of McClintock et al. (14) and Stephenson et al. (personnal communication) similarly indicate that G$_{\text{IX}}$-gp70 belongs to xenotropic virus.

Summary

The family of glycoproteins called gp70 includes molecules that are the main constituent of murine C-type viral envelopes, and some that are expressed as mendelian constituents of thymocyte plasma membranes in the absence of virions. To investigate further the relation of viral gp70s to plasma-membrane gp70s we compared peptide maps of gp70s derived by immunoprecipitation from cells infected with chosen viruses and from various thymocytes and leukemia cells known to express one or more of three immunogenetically defined gp70 types: G$_{\text{IX}}$-gp70, X-gp70, and O-gp70. Maps of gp70 from cultured cells infected with ecotropic and xenotropic viruses were distinguishable from one another, and in general resembled gp70 maps prepared directly from ecotropic and xenotropic virions respectively. Maps of gp70s immunoprecipitated from thymocytes of five mouse strains and from two A strain T-cell leukemias also fell into two distinguishable and generally corresponding patterns. Thus peptide-mapping substantiates earlier conclusions that viral gp70s and plasma-membrane gp70s inherited independently of virus-production are highly related or identical molecules. The gp70 maps of thymocytes from B6, B6-G$_{\text{IX}}^{+}$, 129, and A mice formed a group resembling the map from cultured cells infected with xenotropic virus. Thymocytes from AKR mice, and the two A strain leukemias, gave gp70 maps conforming more to the second pattern, that of cultured cells

infected with ecotropic virus. This second pattern probably comprises at least two gp70 types, one of which is X-gp70. Our data indicate that the G_{IX}-gp70 and O-gp70 sub-species of gp70 expressed in the cell populations we have studied are coded by xenotropic viral genomes, and X-gp70 by ecotropic viral genomes.

We thank Doctors R. A. Lerner for valuable discussions and introducing us to the technique of two-dimensional peptide mapping; J. H. Elder for the goat α-R-MuLV gp70 serum; R. Wilsnack for the pig α-goat-Ig; Ms. N. Gobeo for excellent technical assistance; and Mr. J. Hlinka for the photography.

Received for publication 30 November 1977.

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Brief Definitive Report

SPECIFIC PROTEASE DEFICIENCY IN POLYMORPHONUCLEAR LEUKOCYTES OF CHÉDIAK-HIGASHI SYNDROME AND BEIGE MICE*

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The Chédiak-Higashi syndrome (CHS) is a rare disorder, characterized by giant granules in most granule-containing cells. Its major clinical expression is a marked susceptibility to pyogenic infections which, in the absence of defects in humoral or cellular immunity, has directed attention to polymorphonuclear leukocytes (PMNs). The properties of CHS PMNs include abnormally large azurophile granules (1), somewhat reduced levels of β-glucuronidase and myeloperoxidase (35–50% of normal) (2), defects in chemotactic responses (3), and low bactericidal activity against some gram-positive microorganisms (4). Many of these defects, including those in PMNs, have also been found in animal models such as the beige mouse (bg/bg) (5), mink, cattle (6), cats (7), and a killer whale (8), but no specific molecular defect that could account for the enhanced sensitivity to bacterial infections has been identified.

We recently found that fibrinolysis by human PMNs is due to the combined effects of plasminogen activator and elastase (9). Since elastase is localized in azurophile granules (10), the abnormality of these organelles in CHS leukocytes prompted us to assay for this enzyme. The results show that leukocyte elastase is either very low or undetectable in CHS patients, and the corresponding murine protease is profoundly decreased in leukocytes of beige mice.

Materials and Methods

Materials. NCS mice were obtained from the stock maintained at The Rockefeller University; BALB/cJ and C57 BL/6J from The Jackson Laboratory, Bar Harbor, Maine; and C57 BL/6J bg/bg were the generous gift of Dr. J. Oliver at the University of Connecticut Health Center, Farmington, Conn. Plasmagel was obtained from Roger Bellon Laboratories, Paris, France. The sources of all other materials were as described previously (9).

Case Histories

The patients described below have been studied at the Hôpital des Enfants Malades, Paris, and were in the care of one of the authors (C. G.).

CHS CASE NO. 1. 5-yr-old female, with frequent *Staphylococcus aureus* infections. Leukocytes: 3,200 per mm^3; 50% neutrophils, 99% with abnormal granules; bactericidal activity (11) decreased against *S. aureus*, normal against *Serratia marcescens*. Not in accelerated phase of the disease, no treatment.

CHS CASE NO. 2. 4-yr-old female, rare *S. aureus* infections. Leukocytes: 4,200 per mm^3; 55%

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* Supported in parts by grants from The National Institutes of Health (CA-08290), the American Cancer Society, Inc. (ACS PDT 1H), and The Rockefeller Foundation.

neutrophils, 66% with abnormal granules; bactericidal activity decreased against *S. aureus* and *Escherichia coli*. Not in accelerated phase, no treatment.

CHS CASE NO. 3. 5-yr-old male, frequent *Neisseria* and *Candida albicans* infections. Leukocytes: 3,300 per mm^3, 55% neutrophils, 100% abnormal granules; bactericidal activity decreased against *S. aureus*, normal against *S. marcescens*. Not in accelerated phase of the disease, no treatment.

Preparation of Cells

HUMAN. Human leukocytes were prepared by dextran or plasmagel sedimentation (1 h, 37°C) of heparinized (2 U/ml) venous blood. The plasma containing leukocytes was collected, the cells were washed twice by centrifugation (300 *g*, 5 min) and resuspended in phosphate-buffered saline (PBS). Cells were counted in Türk's solution, and also stained with Giemsa for differential counting. After final centrifugation, the pellet was frozen, stored at −80°C, and transported in dry ice. The cells were thawed and resuspended in PBS containing 0.5% Triton X-100, at 1.5×10^7 PMNs/ml, the lysate was vortexed, centrifuged (1,000 *g*, 10 min), and the supernate was assayed for fibrinolytic activity.

MOUSE. Peritoneal cells were collected by lavage with PBS 16 h after intraperitoneal injection of 30-μg endotoxin (S. Minnesota LPS MR 595-S391) in 1 ml PBS. After washing in PBS, the cells were stained and counted. The yield for all strains tested was $6\text{-}10 \times 10^6$ cells per mouse; 80–85% were PMNs, 10% were macrophages, and 5–10% were lymphocytes. The cells (10^6/ml) were lysed in PBS containing 0.25% Triton X-100, and the lysate was assayed directly, or after dilution in PBS. Alternatively, cells (10^7/ml) were lysed in PBS-0.25% Triton X-100, the lysate was centrifuged (1,000 *g*, 10 min), and the activity of the supernate was analyzed by sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) (9).

Assay for Fibrinolytic Activity. ^{125}I-fibrin-coated wells of Linbro plates (Linbro Scientific Inc., Hamden, Conn.) were prepared as described elsewhere (9). Each well contained ≅20 μg ^{125}I-fibrin (5,000 cpm/μg). The fibrinolytic activity of mouse cells was measured by adding 1 ml of lysates into ^{125}I-fibrin coated wells and measuring the percentage of total radioactivity solubilized during 2 h of incubation at 37°C.

Elastase activity was determined as follows: aliquots (5–50 μl) of human leukocyte lysate or purified porcine pancreatic elastase (0.2 U/ml; 72.7 U/mg, Worthington Biochemical Corp., Freehold, N. J.) were added to ^{125}I-fibrin-coated wells containing 250 μl 0.1 M Tris-Cl, pH 8.1; the rate of fibrinolysis was determined by measuring the radioactivity solubilized at 37°C as a function of time. Under these conditions, the rate of fibrinolysis was linear for up to 2 h, but was not quite linear with concentration of pure elastase or leukocyte extracts. We have therefore used the 2-h values for comparing different samples, and have related these to a standard curve obtained by measuring the activity of known amounts of pure elastase under identical conditions. We note that the specific enzymatic activity of leukocyte preparations may vary, depending upon the purity of PMNs and erythrocyte contamination, and this accounts for the difference in specific activity of PMNs from normal subjects in the present work, compared with that reported previously (9). Finally, crude leukocyte lysates inhibit somewhat the activity of pure elastase, so that the addition of pure enzyme to cell extracts yields values that are not fully additive; the shortfall being ≅30%. We do not know whether this is due to inactivating enzymes, to specific elastase inhibitors, or to substrate competition between leukocyte protein and ^{125}I-fibrin.

Results

Human PMNs contain several proteases that are catalytically active at neutral pH, including collagenase (12), plasminogen activator (9), a chymotrypsin-like enzyme (13), and elastase (14). When human PMNs are lysed and assayed with ^{125}I-fibrin as substrate in the absence of plasminogen, the main source of proteolysis is elastase. In fact, the plasminogen-independent fibrinolytic activity of human leukocyte lysates is a measure of their elastase (9), as suggested by the following evidence: (*a*) the activity is resistant to trypsin and chymotrypsin inhibitors and it is inhibited more than 85% by *N*-acetyl-(Ala)$_4$-chloromethylketone (5×10^{-3} M), a specific inhibitor of elastase; (*b*) the activity migrates in SDS-PAGE as three bands with apparent mol wt in the range

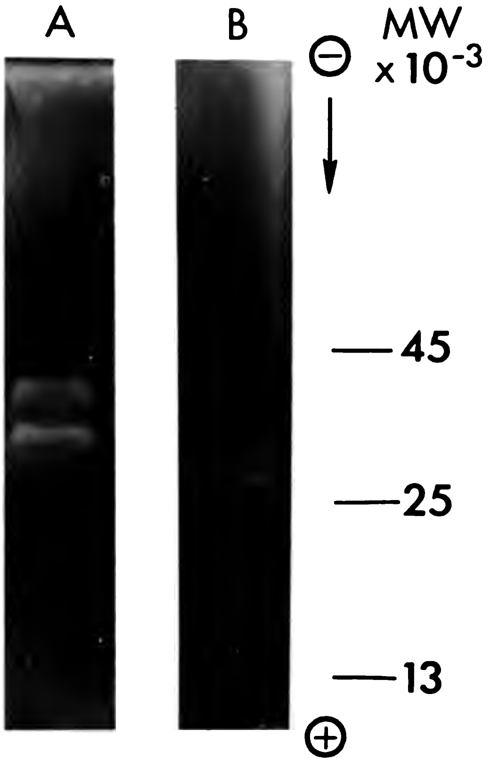

FIG. 1. Identification of proteases after SDS-PAGE of lysates of: (A) human blood leukocytes, and (B) mouse peritoneal exudate leukocytes. Lysates were prepared as described in Materials and Methods, electrophoresed in SDS-11% polyacrylamide slab gels, and the gels were processed for detection of fibrinolytic activity using a fibrin-agar gel (9), in the absence of plasminogen. The figure is a photograph of the amido-black-stained fibrin-agar layer; the clear areas are due to lysis of fibrin in the agar gel by proteases diffusing out of the polyacrylamide gels. Parallel lanes containing lysates of CHS blood leukocytes or beige mouse peritoneal exudate leukocytes did not show any zones of lysis.

28,000–35,000, corresponding to those reported for purified human leukocyte elastase (14); (*c*) when assayed using fibrin-agar gels (9), elastase is the major neutral protease detected in lysates of unfractionated leukocytes from blood (Fig. 1 A).

Murine leukocyte lysates also contain a single major neutral protease when assayed on fibrin-agar gels (Fig. 1 B). In mol wt ≅27,000 this enzyme is close to human elastase, and, like elastase, it is a serine protease inhibited 92% by diisopropyl-fluorophosphate (10^{-4} M) and 87% by soybean trypsin inhibitor (100 μg/ml). However, unlike elastase, it is resistant to *N*-acetyl-$(\text{Ala})_4$-chloromethylketone; it is also resistant to the trypsin inhibitors *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (10^{-3} M) and nitrophenyl-*p*-guanidinobenzoate (10^{-4} M). The chymotrypsin inhibitors L-1-tosylamide-2-phenylethyl-chloromethyl ketone (10^{-3} M) and *N*-carbobenzoxy-L-phenylalanine chloromethyl ketone (10^{-3} M) reduced this activity by somewhat less than 50%. Thus, this enzyme is a serine protease of the pancreatic type, but its precise substrate specificity, while in part chymotryptic, remains incompletely defined.

We have tested lysates of buffy coats from CHS patients, and of peritoneal

TABLE I

Elastase in Lysates of Human Leukocytes

CHS case no.	Elastase (units per 10^6 PMNs × 10^3)
1	<0.1
2	0.2
3	<0.1
Normal (range)	3.2 (1.5–5.6)

Human leukocytes were prepared, lysed, and assayed for fibrinolytic activity, and the values obtained were related to standard preparations of purified elastase, as described in Methods. The elastase content of normal leukocytes was determined by assaying cells from six control subjects (two adults and four children).

exudates from beige mice both by the gel method (Fig. 1), and by measuring the solubilization of ^{125}I-fibrin. In the gel system elastase was undetectable, and no other plasminogen-independent activity was present in human CHS lysates; likewise, the corresponding murine protease (of Fig. 1 B) was absent in lysates of beige mouse peritoneal leukocytes. When assayed with ^{125}I-fibrin as substrate, plasminogen-independent fibrinolysis was undetectable in lysates from two CHS patients, whereas the third patient, clinically the least affected, contained less than 10% of the normal level (Table I). Comparable results for mice are presented in Table II, and these show that lysates from beige mice contain at most 3% of the neutral protease activity found in control strains.

It is unlikely that the reduced activity in either of the affected cell extracts is due to excess inhibitors. Mixtures of inactive and active extracts gave some reduction of the expected level, but this was slight (35%). More importantly, however, we have found that all catalytically inactive complexes formed between proteases and macromolecular inhibitors so far tested are dissociated during SDS-PAGE, allowing the protease to be visualized by the gel method of Fig. 1 (A. Granelli-Piperno and E. Reich, unpublished observations). Were an inactive enzyme-inhibitor complex the explanation for our findings, some active bands should have been detected in the gels. Although we cannot rigorously exclude excess inhibitor as the basis of our observations, such a mechanism is not the most probable one.

Discussion

Despite the limitations of the procedures noted in Materials and Methods, our results leave little doubt that CHS leukocytes are profoundly deficient in elastase, and that an analogous deficiency occurs in beige mice. Moreover, since elastase is localized in azurophile granules, our findings provide a satisfying correlate for the morphological abnormality of these granules both in CHS and in beige mice. While no cause and effect relationships between the enzymatic and morphological changes can be deduced at present, it will be of interest to determine whether or not specific enzyme deficiencies are associated with the granule alterations in other tissues. Since the enzyme deficiency we have observed could arise from failure to activate proenzymes, or from irreversible enzyme inactivation during granule formation, it would also be useful to assay CHS and beige mouse leukocytes for substances that might be immunologically cross-reactive with their respective missing enzymes.

Of the three CHS patients examined, two (case nos. 1 and 3) were severely

TABLE II

Proteolytic Activity of Lysates of Mouse Peritoneal Cells

Strain	Substrate solubilized by lysates from:	
	3×10^4 Cells	1×10^6 Cells
	%	
BALB/cJ	4.7	37
NCS	1.9	31
C57 BL/6J, +/+	2.4	33
C57 BL/6J, bg/bg	0.1	2.3

Peritoneal cells from endotoxin-injected mice were lysed and assayed for fibrinolytic activity as described in Materials and Methods.

affected and experienced numerous infections; all of their PMNs had abnormal granules, and their leukocyte elastase was undetectable. A third patient (case no. 2) had a clinically milder form of the disease; abnormal granules were present only in a fraction of her PMNs, and her level of elastase was very low, but detectable. Hence, severity of disease, proportion of morphologically affected PMNs, and levels of leukocyte elastase appear to be correlated; it will be useful to test this correlation in a larger series of CHS patients.

The increased susceptibility of CHS patients to infections has been linked to the impairment of leukocyte function, and the reported defects in bactericidal capacity of PMNs from CHS patients (4) and beige mice (5) might be significant pathogenetic factors. Since normal phagocytic rate and capacity of PMNs are retained in these disorders (15, 5), the reduced bactericidal activity is more likely due to a defective intracellular killing mechanism. The possibility that elastase deficiency might introduce a rate limitation in the killing of some bacteria is attractive, because the peptidoglycan structure of organisms such as *S. aureus* contains an elastase-sensitive bond (16), and because this enzyme can lyse the cell walls of autoclaved *S. aureus* (16), a pathogen that CHS PMNs kill inefficiently (4), and to which these patients are specially susceptible (17). While not by itself bactericidal in vitro (16), elastase potentiates the activity of granulocyte microbicidal systems against *E. coli* and *S. aureus* (18), and participates in the digestion of *E. coli* proteins in human PMNs (19); elastase might therefore be essential for normal rates of intracellular killing.

Summary

Peripheral blood leukocytes of three patients with Chédiak-Higashi syndrome (CHS) contained very low or undetectable levels of elastase, the major neutral protease in these cells. Likewise, peritoneal exudate leukocytes of beige mice (the murine counterpart of CHS) contained correspondingly reduced levels of their major neutral protease, a serine enzyme of mol wt 27,000. The elastase deficiency in CHS polymorphonuclear leukocytes might account in part for the high incidence of infections in these patients.

We thank Ms. Anne McGinley, Ms. Virginia Iliescu, and Mrs. Atsuko Horiuchi for their excellent technical assistance, and Dr. J. Oliver for stimulating discussions and generous gift of beige mice.

Received for publication 1 November 1977.

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Brief Definitive Report

CYTOTOXIC T CELLS RECOGNIZE MALE ANTIGEN AND H-2 AS DISTINCT ENTITIES

BY HARALD VON BOEHMER, WERNER HAAS, AND HELMUT POHLIT

(From the Basel Institute for Immunology, Basel 5, Switzerland)

Female mice of some strains are capable of mounting cytotoxic T-cell responses to male cells. The genes determining responsiveness are *H-2*-linked (1, 2). Such cytotoxic T cells discriminate between male targets expressing different H-2 antigens, i.e. they are restricted by H-2 (1, 2). The effector cells may either recognize the Y-antigen and H-2 as two distinct entities, or they may recognize new antigenic determinants formed when the Y-antigen is associated with H-2.

In line with the second alternative, it has been proposed that H-2 antigens are the anchorage site for Y-antigens: to explain virilization of XX bovine freemartin gonads, Ohno and Martin (3, 4) assumed that XY cells from the bull twin disseminate Y-antigen, coating the majority of XX cells.

We report herein tests for the presence of male-specific determinants on XX cells from XX/XY hemopoietic chimeras, and on the reactivity of male or female T cells against *H-2*-different male cells.

Materials and Methods

Cytotoxic Anti-Male Responses. Mice were immunized by intravenous injection of 2 × 10^7 XY spleen cells irradiated with 2,200 R. From these mice, spleen cells were prepared 2–20 wk later, and cultured for 5 days with irradiated XY spleen cells (2). Cytotoxic tests were performed on ^{51}Cr-labeled blasts induced by lipopolysaccharide (LPS) (2, 5).

Chimeras. Chimeras were prepared by injecting lethally (880 R) irradiated female (CBA/J × C57Bl/6)F_1 hybrids with anti-θ-treated male or female bone marrow cells from one or both parental strains (5). Lymphoid cell chimerism was checked by using cytotoxic anti-H-2 sera (5). Some of the chimeras were immunized with male cells 3 mo after bone marrow reconstitution.

Depletion of Alloreactive T Cells. Recirculating lymphocytes specifically depleted of alloreactive T cells were obtained by the method of Sprent and Miller (6). Allogeneic spleen cells were irradiated with 1,000 R, cultured for 5 h in vitro, and then 4 × 10^8 cells were injected intravenously. Thoracic duct lymphocytes were collected 24–48 h after injection.

Results

XX Cells from XX/XY Chimeras Are Not Lysed by Male-Specific Effector Cells. XX/XY hemopoietic chimeras were produced by injecting equal numbers of XX or XY CBA/J and XY or XX C57Bl/6 bone marrow cells, respectively, into lethally irradiated female (CBA/J × C57Bl/6)F_1 hybrids. Lymphoid cell chimerism in the spleen was tested 3 mo later, and ranged in the different chimeras from 20 to 30% C57Bl/6 cells, and from 70 to 80% CBA/J cells. Persisting F_1 hybrid cells could not be demonstrated.

It was then tested whether only XY or also XX cells from such chimeras could be lysed by *H-2*-restricted cytotoxic lymphocytes recognizing Y-antigens. Targets were prepared by stimulating spleen cells from XX/XY chimeras with LPS and labeling the cells with ^{51}Cr. *H-2^b*-restricted cytotoxic T cells were obtained

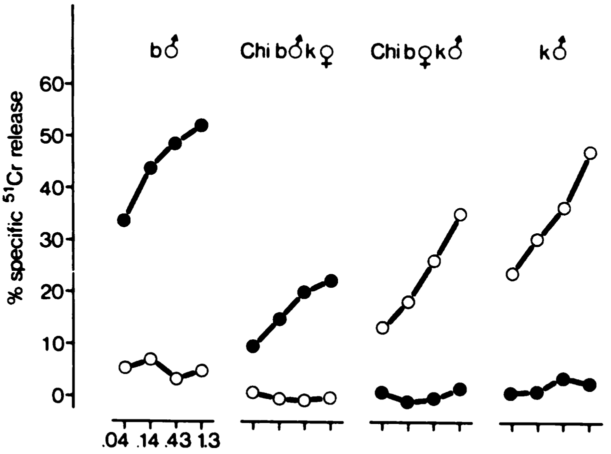

FIG. 1. Lysis of C57Bl/6 ♂ (b♂), CBA/J ♂ (k♂), and b♂ k♀ or b♀ k♂ targets from chimeric mice by anti-male killer cells restricted to H-2^b (●) or to H-2^k (○). The abscissa shows the numbers (× 10^6) of female responder cells cultured on day 0, the descendants of which are the cytotoxic cells producing lysis on day 5 of culture.

from cultures of female C57Bl/6 cells stimulated with *H-2*^{b} male cells, *H-2*^{k}-restricted effector cells from cultures of female (C57Bl/6 × CBA)F$_1$ cells stimulated with male CBA/J cells. (CBA/J mice cannot be sensitized to kill XY *H-2*^{k} cells). The two effector cell populations were then tested on various targets. As shown in Fig. 1, *H-2*^{b}-restricted killer cells lysed only targets containing XY *H-2*^{b}, but not targets containing XX *H-2*^{b} and XY *H-2*^{k} cells. The reciprocal result was obtained with *H-2*^{k}-restricted effector cells. Thus, no Y-antigens were detected on XX cells in XX/XY hemopoietic chimeras. The fact that XY hemopoietic cells from XX recipient mice were lysed, indicates that the Y-antigen recognized by cytotoxic lymphocytes was an endogenous product of the target cells.

The Ability of Cells from XX/XY Chimeras to Induce Cytotoxic Responses. Results like those described in the preceding section were obtained when the stimulatory capacity of cells from XX/XY chimeras was tested. Female (CBA/J × C57Bl/6)F$_1$ cells primed in vivo with either CBA/J or C57Bl/6 XY cells were mixed in equal proportions. Such cells stimulated with XY *H-2*^{k} or XY *H-2*^{b} cells produced cytotoxic effector cells restricted to H-2^k or H-2^b, respectively (Fig. 2). A mixture of XY *H-2*^{b} and XX *H-2*^{k} cells from either chimeric or normal mice induced male-specific effector cells restricted to H-2^b, whereas a mixture of XX *H-2*^{b} and XY *H-2*^{k} cells stimulated an *H-2*^{k}-restricted response (Fig. 2), indicating once again that Y-antigen was not recognized on female cells.

The Cytotoxic Response of Female or Male H-2^b *T Cells to Syngeneic or Allogeneic Male Cells.* XX *H-2*^{b} T cells from a chimera, produced by injecting XX C57/Bl/6 bone marrow into lethally X-irradiated XX (C57Bl/6 × CBA/J)F$_1$ hybrids, can be sensitized to kill both *H-2*^{b} and *H-2*^{k} XY targets (Fig. 3). Thus, as previously reported for 2,4,6-trinitrophenyl (TNP) (7, 8) and for viral antigens (8, 9), the response to male antigens in chimeras can be restricted to both *H-2*^{b} of the responder and *H-2*^{k} of the recipient strain, even though the *H-2*^{k} strain

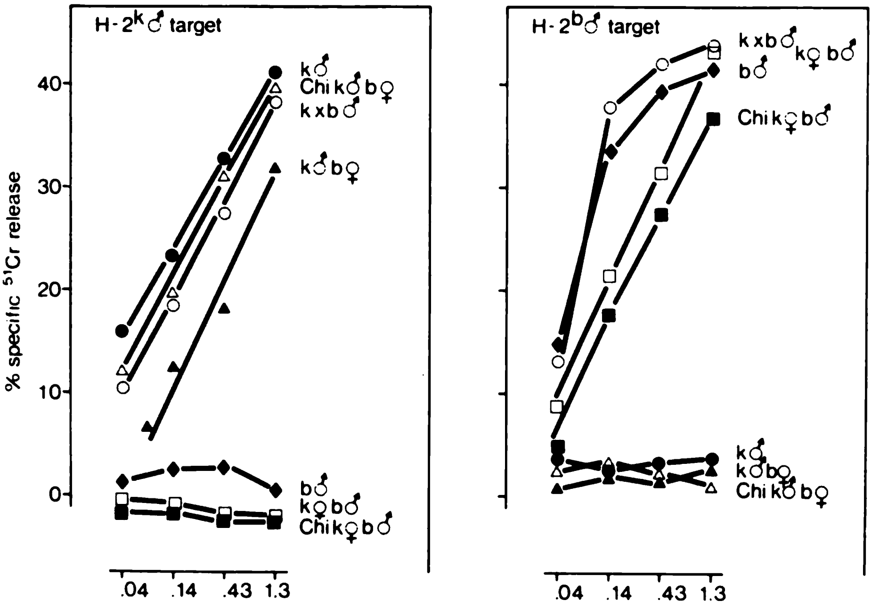

FIG. 2. Stimulation of *H-2*-restricted cytotoxic effector cells from primed female (CBA/J × C57Bl/6)F$_1$ hybrids by C57Bl/6 ♂ (b♂), CBA/J ♂ (k♂), (CBA/J × C57Bl/6)F$_1$ ♂ (k × b♂), and by a mixture of k♂ and b♀ (k♂ b♀), or k♀ and b♂ (k♀ b♂) cells from either normal or chimeric mice.

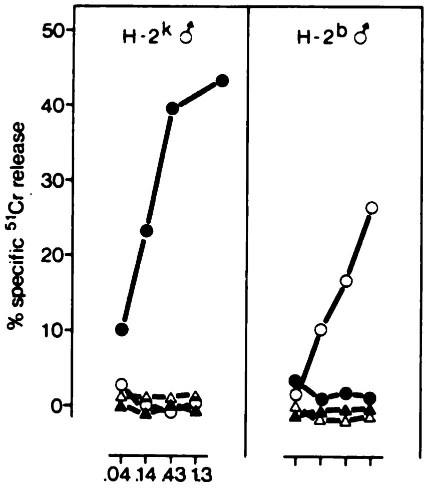

FIG. 3. Lysis of male H-2^k or H-2^b targets by T cells from C57Bl/6 ♀ → (CBA/J × C57Bl/6)F$_1$ ♀ (circles) or C57Bl/6 ♂ → (CBA/J × C57Bl/6)F$_1$ ♀ chimeras (triangles) stimulated in vitro by either C57Bl/6 ♂ (○, △) or by CBA/J ♂ (●, ▲) stimulators.

itself does not mount an anti-Y response. However, XY *H-2^b* T cells from a chimera, produced by injecting C57Bl/6 XY bone marrow into XX (C57Bl/6 × CBA/J)F$_1$ recipients, cannot be educated to kill either *H-2^b* or *H-2^k* male cells (Fig. 3), while responding to allogeneic cells (not shown).

As shown in Fig. 3, XX *H-2^b* T cells from chimeras could be sensitized to lyse XY *H-2^k* targets. The same could not be demonstrated with XX *H-2^b* T cells

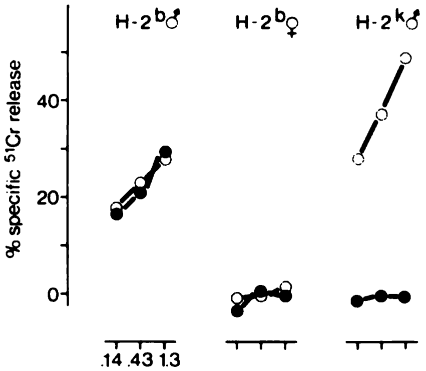

FIG. 4. Lysis of XX and XY H-2^b and XY H-2^k targets by XX H-2^b TDL negatively selected to H-2^k alloantigens (●), or XX *H-2^b* T cells (○) stimulated by XY (CBA/J × C57Bl/6)F_1 cells. The mice had been primed in vivo with XY (CBA/J × C57Bl/6)F_1 cells.

depleted of alloreactive T cells from normal mice. For this experiment, XX C57Bl/6 mice were primed in vivo with XY (CBA/J + C57Bl/6)F_1 cells, and injected intravenously 14 days later with irradiated (1,000 R) XX (CBA/J × C57Bl/6)F_1 cells, to recruit T cells reactive to H-2^k alloantigens to the spleen (6). Thoracic duct lymphocytes (TDL) were collected between 24 and 48 h after injection of XX F_1 cells. The TDL were then cultured with XY (CBA/J × C57Bl/6)F_1 stimulators.

As shown in Fig. 4, the TDL could be activated to lyse XY *H-2^b* targets, but not XY *H-2^k* targets. In control cultures, XY-primed *H-2^b* T cells were stimulated with XY (CBA/J × C57Bl/6)F_1 cells. These cells lysed XY *H-2^b* targets and, more effectively, allogeneic XY *H-2^k* as well as XX target cells (not shown).

Discussion

The experiments reported here do not support the idea that male antigens associated with H-2 form new antigenic determinants recognized by T cells. XX cells from XX/XY hemopoietic chimeras do not take up male antigens disseminated by XX cells in a way which would allow them to serve as stimulators or targets for male-specific killer cells in vitro. Nor is it likely that new antigenic determinants are formed when male antigens and H-2 are produced and expressed by the same cell. In that case, *H-2^b* male cells tolerant to H-2^k antigens should, like *H-2^b* female T cells, be sensitized to lyse male *H-2^k* cells. Our experiments do not entirely rule out the possibility that complexes of disseminated male antigens and H-2 exist on stimulator and target cells in quantities below the level of detection, but sufficient to induce tolerance. Even if nonreactivity of XY T cells to allogeneic XY cells could be explained in this fashion, there is no explanation why XX *H-2^b* T cells negatively selected to H-2^k alloantigens should not be able to recognize and react to new antigens formed by Y-antigens and H-2^k alloantigen. (The negative result of this experiment is in contrast to the findings of Wilson et al. [10] that negatively selected T cells could be activated to kill allogeneic TNP-coupled target cells. The discrepancy would suggest that T cells can be activated to certain haptens coupled directly to alloantigens).

Our experiments imply that T cells distinguish between male cells expressing different H-2 antigens, and that they do this by recognizing H-2 and the Y-antigen as distinct entities.

But this still leaves open the role of the two entities in activating cytotoxic cells. The fact that XX T cells from a chimera but not those from a normal mouse can respond to Y-antigens on allogeneic cells suggests that T cells in a chimera acquire the potential of recognizing Y-antigens in association with allogeneic H-2. Recent experiments by Zinkernagel et al. (11) indicate that in the thymus of a chimera, T cells do not only become tolerant to H-2 (5), but they also learn which H-2 type to use in interaction with other cells.

Summary

XX cells from XX/XY hemopoietic chimeras do not express male determinants in a way to render them either stimulators or targets for male-specific cytotoxic lymphocytes. XX- but not XY-responder T cells from chimeras can be activated to lyse allogeneic male target cells; T cells from normal XX mice depleted of alloreactive T cells, however, cannot be sensitized to lyse allogeneic XY targets. The results imply that T cells recognize the Y-antigen and H-2 as distinct entities, and that in chimeras, they acquire the potential to react against allogeneic XY cells.

Received for publication 2 December 1977.

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3 Pang N. Shek and Albert H. Coons. Effect of colchicine on the antibody response. I. Enhancement of antibody formation in mice

3 Pang N. Shek, Carl Waltenbaugh, and Albert H. Coons. Effect of colchicine on the antibody response. II. Demonstration of the inactivation of suppessor cell activities by colchicine

T. J. Braciale and K. L. Yap. Role of viral infectivity in the induction of influenza virus-specific cytotoxic T cells

Ben A. M. van der Zeijst, Carleton C. Stewart, and Sondra Schlesinger. Proliferative capacity of mouse peritoneal macrophages in vitro

B. J. Mathieson, P. S. Campbell, M. Potter, and R. Asofsky. Expression of Ly 1, Ly 2, Thy 1, and TL differentiation antigens on mouse T-cell tumors

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CONTENTS THE JOURNAL OF EXPERIMENTAL MEDICINE

Volume 147 No. 4 April 1, 1978

963 S. Slavin, Z. Fuks, H. S. Kaplan, and S. Strober. Transplantation of allogeneic bone marrow without graft versus host disease using total lymphoid irradiation

973 Vanda A. Lennon, Marjorie E. Seybold, Jon M. Lindstrom, Charles Cochrane, and Richard Ulevitch. Role of complement in the pathogenesis of experimental autoimmune myasthenia gravis

984 Samuel J. Black, Wessel van der Loo, Michael R. Loken, and Leonard A. Herzenberg. Expression of IgD by murine lymphocytes. Loss of surface IgD indicates maturation of memory B cells

997 Judith A. Kapp. Immunosuppressive factors from lymphoid cells of nonresponder mice primed with L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}. IV. Lack of strain restrictions among allogeneic, nonresponder donors and recipients

1007 Charles E. Davis, Elizabeth J. Ziegler, and Karen F. Arnold. Neutralization of meningococcal endotoxin by antibody to core glycolipid

1018 Margaret A. Alspaugh, Fred C. Jensen, Harvey Rabin, and Eng M. Tan. Lymphocytes transformed by Epstein-Barr virus. Induction of nuclear antigen reactive with antibody in rheumatoid arthritis

1028 Kenji Okuda and Chella S. David. A new lymphocyte-activating determinant locus expressed on T cells and mapping in *I-C* subregion

1037 Edgar G. Engleman, Andrew J. McMichael, and Hugh O. McDevitt. Suppression of the mixed lymphocyte reaction in man by a soluble T-cell factor. Specificity of the factor for both responder and stimulator

1044 Charles D. Scher. Effect of pseudotype on Abelson virus and Kirsten sarcoma virus-induced leukemia

1054 Svante Stenman and Antti Vaheri. Distribution of a major connective tissue protein, fibronectin, in normal human tissues

1065 Charles A. Janeway, Jr., Patricia D. Murphy, John Kemp, and Hans Wigzell. T cells specific for hapten-modified self are precommitted for self major histocompatibility complex antigens before encounter with the hapten

1078 Edward F. Voelkel, Lawrence Levine, Chester A. Alper, and Armen H. Tashjian, Jr. Acute phase reactants ceruloplasmin and haptoglobin and their relationship to plasma prostaglandins in rabbits bearing the VX$_2$ carcinoma

1089 Yuichi Obata, Elisabeth Stockert, Paul V. O'Donnell, Shuji Okubo, Harry W. Snyder, Jr., and Lloyd J. Old. G$_{(RADA1)}$: a new cell surface antigen of mouse leukemia defined by naturally occurring antibody and its relationship to murine leukemia virus

1106 D. D. Eardley, J. Hugenberger, L. McVay-Boudreau, F. W. Shen, R. K. Gershon, and H. Cantor. Immunoregulatory circuits among T-cell sets. I. T-helper cells induce other T-cell sets to exert feedback inhibition

1116 H. Cantor, L. McVay-Boudreau, J. Hugenberger, K. Naidorf, F. W. Shen, and R. K. Gershon. Immunoregulatory circuits among T-cell sets. II. Physiologic role of feedback inhibition in vivo: absence in NZB mice

1126 Naomi Rosenberg and David Baltimore. The effect of helper virus on Abelson virus-induced transformation of lymphoid cells

1142 J. Sprent. Restricted helper function of F$_1$ hybrid T cells positively selected to heterologous erythrocytes in irradiated parental strain mice. I. Failure to collaborate with B cells of the opposite parental strain not associated with active suppression

1159 J. Sprent. Restricted helper function of F$_1$ hybrid T cells positively selected to heterologous erythrocytes in irradiated parental strain mice. II. Evidence for restrictions affecting helper cell induction and T-B collaboration, both mapping to the *K*-end of the *H-2* complex

1175 Saimon Gordon and Zanvil A. Cohn. Bacille Calmette-Guérin infection in the mouse. Regulation of macrophage plasminogen activator by T lymphocytes and specific antigen

1189 Mary A. Maloney, Mary J. Dorie, Rosito A. Lamela, Zora R. Rogers, and Harvey M. Patt. Hematopoietic stem cell regulatory volumes as revealed in studies of the bg^J/bg^J: W/W^v chimera

CONTENTS CONTINUED ON REVERSE OF THIS COVER

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VOLUME 147, No. 5 MAY 1, 1978

JEMEAV 147(5) 1299-1534 (1978) • ISSN 0022-1007

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ANTI-VIRAL ACTIVITY INDUCED BY CULTURING LYMPHOCYTES WITH TUMOR-DERIVED OR VIRUS-TRANSFORMED CELLS

Identification of the Anti-Viral Activity as Interferon and Characterization of the Human Effector Lymphocyte Subpopulation*

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Interferons are cellular glycoproteins that inhibit viral replication and that may be involved in the defensive response of an animal to virus infection. Virus infection of cells induces the synthesis and release of interferons, which render uninfected cells resistant to virus infection. Interferon can be induced by agents other than viruses, such as micro-organisms (1), substances of microbial origin (2), and synthetic polymers (3). Although interferon is not specific for a particular virus, it displays a characteristic cell specificity, being more active on homologous than on heterologous cells. Interferons not only display anti-viral activity, but also have been shown to affect various cellular mechanisms that are not directly related to viral replication. Interferons inhibit cell growth (4), facilitate or suppress interferon induction (priming and blocking effects) (5, 6), and increase the susceptibility of cells to the toxicity of double-stranded RNAs (7). Moreover, they affect several immunological functions: interferons inhibit antibody formation in vivo and in vitro (8, 9), and enhance the phagocytic activity of macrophages (10), the cytotoxicity of lymphocytes (11-13), and IgE-mediated histamine release from basophils (14).

Exposure of lymphocytes to viruses induces the release of interferon molecules that can be distinguished immunologically from the interferon produced by other cell types (15). The synthesis of interferon from lymphocytes can also be induced by immune stimulation (bacterial [16] and viral antigens [17-19], allo-antigens [20]), anti-lymphocyte sera (21), and mitogenic lectins (22).

We have recently described that viral inhibitors are released in the supernates of mixed cultures of lymphocytes and certain tumor-derived or virus-transformed cell cultures (23). In the present study we identify these inhibitors

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* Supported, in part, by U. S. Public Health Service research grants CA20833, CA10815, CA43882, and CA18470 from the National Cancer Institute, NS11036 from the National Institute of Neurologic and Communicative Diseases and Strokes, CDA AI00053 from the National Institute of Allergy and Infectious Diseases (BBK), IM-88 from the American Cancer Society, and by the National Multiple Sclerosis Society.

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J. Exp. Med. © The Rockefeller University Press · 0022-1007/78/0501-1299$1.00

as interferons and characterize some of their anti-viral and anti-cellular activities. Moreover we identify the producer cells in the human peripheral blood as Fc-receptor bearing lymphocytes that have no surface immunoglobulins and that do not form rosettes with sheep erythrocytes.

Materials and Methods

Reagents. The reagents used in these studies and their sources are as follows: cycloheximide, 2-aminoethylisothio-uronium bromide hydrobromide (AET),[1] and trypsin crystallized two times from bovine pancreas (3.4.4.4) from Sigma Chemical Co., (St. Louis, Mo.); actinomycin D from Calbiochem (San Diego, Calif.).

Cell Lines and Virus. The origin of and reference for the cell lines used in this study are summarized in Tables I and II. Most of the simian virus 40 (SV40)- and adenovirus 5-transformed cell lines were originated in our laboratories.

The 73-T strain of Newcastle disease virus (NDV) (allantoic fluid, 3.3 × 10^9 plaque-forming units (PFU)/ml on L929 cells), the Indiana strain of vesicular stomatitis virus (VSV, 3 × 10^8 PFU/ml on L-F_2 cells) and encephalomyocarditis virus (EMC, 4 × 10^8 PFU/ml on F-F_2 cells) were originally obtained from T. J. Wiktor (Wistar Institute). Vaccinia virus (Lister strain), provided by M. Herlyn (Wistar Institute), was originally obtained from Wyeth Laboratory, (10^6 TCID50/ml on WI38 cells). The Hong Kong/107/68 (H3N2) strain of influenza A virus (allantoic fluid, 850 hemagglutination units-ml) was obtained from W. Gerhard (Wistar Institute).

Human Lymphocyte Separation. Lymphocytes were obtained from heparinized human peripheral blood by centrifugation on a Ficoll-Hypaque gradient (31). The lymphocyte suspensions obtained was contaminated by 5–30% monocytes as identified by nonspecific acid esterase staining (acid α-naphthyl acetate esterase, ANAE) (32) and was further depleted of adherent cells by two 1-h incubations on glass Petri dishes at 37°C.

Further cell fractionation was obtained by separation of nylon fiber-adherent cells (33).

Cells bearing surface immunoglobulins or Fc-receptors were removed by passing the lymphocyte population through a Sepharose IgG-anti-IgG column. Cyanogen bromide-activated Sepharose 6B (Pharmacia, Uppsala, Sweden) was conjugated with normal human IgG (purified by DEAE chromatography, 10 mg/ml of gel), and columns containing 6 ml gel were prepared. 20 mg of IgG from a polyvalent rabbit anti-human IgG serum was run on the column, which was eluted several times before 10^8 lymphocytes in RPMI 1640 medium (1% FBS) were added to it and incubated 30 min at 4°C. The nonadherent cells were eluted from the column with ice-cold medium. Columns without anti-IgG serum were used as controls.

Cells forming rosettes with AET-treated sheep erythrocytes were separated from non-rosette-forming cells by the method of Pellegrino and Ferrone (34).

Mixed Cultures. Tumor-derived, virus-transformed, and fibroblast cell monolayers were grown in 16-mm wells (Disposo Trays FB16-24TC, Linbro Scientific, Inc. Div. of Flow Lab., Hamden, Conn.). Lymphocytes depleted of glass adherent cells were resuspended at 10^7/ml in RPMI-1640 medium supplemented with 10% fetal bovine serum (RPMI-FBS, Flow Laboratories, Inc., Rockville, Md.) and 1 ml was added to each well. The mixed cultures were incubated for 24 h at 37°C in a humidified 5% CO_2:95% air atmosphere, then the supernate was collected, centrifuged at 1,000 *g* for 30 min, and stored at −80°C until tested.

Interferon Tests. For testing human interferon, monolayers of fetal skin fibroblasts (FS2) were grown in the wells of microtiter plates (3040, BioQuest, BBL & Falcon Products, Becton, Dickinson & Co., Cockeysville, Md.). The medium was removed from the wells, and 0.1-ml samples of serial dilutions of the supernates to be tested were added. After an 18-h incubation, the supernates were removed and 0.2 ml of VSV (50,000 PFU) was added to each well. The anti-viral activity was measured by inhibition of the cytopathic effect of VSV. Anti-viral units are expressed as the reciprocal of the highest dilution inhibiting 50% of the cytopathic effect. Each assay included a standard interferon preparation (NIH human Reference Interferon G-023-901-527 with a titer of 20,000 U). 1 anti-viral U in our testing procedure is equivalent to approximately 1 reference U of the NIH interferon.

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[1] *Abbreviations used in this paper*: AET, 2-aminoethylisothio-uronium bromide hydrobromide; ANAE, acid alphanaphthyl acetate esterase; EBV, Epstein-Barr virus; EMC, encephalomyocarditis virus; FBS, fetal bovine serum; HAU, hemagglutination units; MOI, multiplicity of infection; NDV, Newcastle disease virus; PBS, phosphate-buffered saline; PFU, plaque-forming unit; RDMC, rhabdomyosarcoma cell line; SV40, simian virus 40; $TCID_{50}$, tissue culture infectious dose 50%; VSV, vesicular stomatitis virus.

Mouse interferon was similarly measured on monolayers of murine L-F_2 cells, by using EMC (50,000 PFU/well) as the challenge virus (35). Assays were standardized against an NIH mouse interferon reference standard. 1 anti-viral U in this testing procedure cells is equal to 1 U of reference standard.

Leucine, Uridine, and Thymidine Uptake. 10^4 trypsinized cells in 0.1 ml RPMI-FBS were added to flat bottom wells of microtiter plates (3040, Falcon), and 0.1 ml of dilutions of the supernates to be tested were added to triplicate wells. The cells were incubated for 18 h at 37°C, then 2 μCi of [^{3}H]labeled precursors (New England Nuclear Corp., Boston, Mass.) were added to each well. The cells were incubated for another 6 h, then the medium was removed, 0.2 ml of trypsin (0.25%) was added, and the cells were collected on to glass fiber filters (GF/B, Whatman, Inc., Clifton, N. J.) by means of a semi-automatic collector (O. Hiller, Madison, Wis.). Radioactivity was assessed by liquid scintillation spectrometry. [^{3}H]thymidine uptake inhibitory units are defined at the reciprocal of the dilution inhibiting 50% [^{3}H]thymidine uptake in rhabdomyosarcoma (RDMC) cells.

Results

Anti-Viral Activity in the Supernates of Mixed Cultures. The results obtained by testing for presence of anti-viral activity in the supernates of mixed cultures of human lymphocytes with cells obtained from fibroblast, virus-transformed, tumor-derived, and lymphoid cell lines are summarized in Table I. No anti-viral activity was usually observed in the supernates from human lymphocytes cultured alone. None of the 13 human fibroblast cultures tested induced anti-viral activity when mixed with human lymphocytes, whereas 13 out of 21 tumor-derived cell lines were inductive. The association between the tumor origin of a line and its ability to induce interferon was highly significant (P = 0.00022, Fisher's exact test). None of the cell lines, including the lymphoid lines, spontaneously released detectable anti-viral activity when cultured alone under conditions comparable to those of the mixed cultures. Moreover, the supernates from inducer cell lines, which included one EBV- and two SV40-producing lines, were unable to stimulate human lymphocytes to produce the virus inhibitor(s).

Table II summarizes the results of culturing murine cells from several different lines with human lymphocytes or with mouse spleen cells. The supernate from BALB/c spleen cells cultured alone usually contained 1-2 U of anti-viral activity. The lines that induced anti-viral activity when cultured with human lymphocytes also induced such activity in mouse spleen cells, although the activity obtained with mouse lymphocytes was always much lower.

Allogeneic and Syngeneic Mixed Cultures. As a control in each experiment, two allogeneic human lymphocyte preparations were cultured together. When tested for anti-viral activity after a 24-h incubation, the supernates from these cultures were always negative. Lymphocytes from donor H. K., from whom the lymphoid line HK was established by transformation of peripheral blood lymphocytes with Epstein-Barr virus (EBV), were able to produce anti-viral activity when mixed with autologous HK cells. When spleen cells from C57BL/6 or BALB/c mice were cultured with cells from lines able to induce interferon production in human lymphocytes, anti-viral activity was detected in the supernate of xenogeneic, allogeneic, and syngeneic combinations (Table III). The anti-viral inhibitors in the supernates from mixed cultures of lymphocytes and

TABLE I

Origin of Human Cells and Ability to Induce Anti-Viral Activity When Cultured with Human Lymphocytes

Cell	Reference	Origin	Passages	Anti-viral Units Induced[*] (Mean ± standard error)
		Fibroblasts		
FS1 and 2		Fetal skin	14-20	<1
Two strains		Fetal skin	35-45	<1
Seven strains		Skin	5-20	<1
Two strains		Brain	2-15	<1
		SV40-transformed		
Five lines		Skin	24-255	<1
Nine lines		Brain	4-22	<1
S1054TR		Brain	32-37	193 ± 137
S10033WTR		Brain	8-12	125 ± 0
		Adenovirus 5-transformed		
293/31	24			<1
NP1	24	293/31 tumor in nude mouse		<1
		Kirsten murine sarcoma virus-transformed (nonproducer)		
2-970-S	25	Osteosarcoma (TE85, Cl F5)		337 ± 152
A172-10[‡]		Melanoma		1,562 ± 587
		Tumor-derived lines		
SW690[§]		Melanoma	80-90	850 ± 552
SW691		Melanoma	80-85	6,000 ± 3,000
SW843		Melanoma	30-40	244 ± 119
SW489		Melanoma	30-40	25 ± 0
A375		Melanoma	76	25 ± 0
A375		Melanoma	95	194 ± 137
Four lines	26	Colorectal carcinoma	10-95	<1
SW620	26	Colorectal carcinoma	160-165	362 ± 137
SW480	26	Colorectal carcinoma	105-110	125 ± 0
D98 (HeLa)	27	Cervical carcinoma	50-60	312 ± 165
A673		Sarcoma		1,081 ± 649
HT1080	28	Fibrosarcoma	110-115	<1
SW80		Rhabdomyosarcoma	48-50	<1
RDMC	29	Rhabdomyosarcoma	150-200	3,494 ± 1,008
TE85, Cl F5	25	Osteosarcoma	15	<1
		Lymphoid lines		
Daudi	30	Burkitt lymphoma		<1
SB		Lymphatic leukemia		1,562 ± 823
PGIP7		Lymphatic leukemia		1,800 ± 693
CMG		PBL-EBV[‖]		75 ± 28
EB-P8		PBL-EBV		2,120 ± 559
HK		PBL-EBV		600 ± 0
(B95)		(Marmoset, EBV producer)		1,800 ± 693

[*] Mean of results obtained with at least four lymphocyte preparations from different donors.

[‡] Cell lines with A as first letter originated in Dr. S. A. Aaronson's laboratory (National Cancer Institute, Viral Carcinogenesis Branch).

[§] Cell lines with SW as first letters originated in Dr. A. Leibovitz' laboratory (Scott and White Clinic, Temple, Texas).

[‖] Human peripheral blood lymphocytes, Epstein-Barr virus-transformed.

TABLE II

Origin of Murine Cells and Ability to Induce Anti-Viral Activity When Cultured with Lymphocytes

Cell line	Mouse strain	Origin	Passages	Anti-viral units produced	
				Human lymphocytes	Mouse lymphocytes
BICR	Random bred albino	Brain	17-19	<1	ND*
3T3	BALB/c	Embryo	97-101	65	ND
NTG2	BALB/c	Embryo (3T3)	80-85	16	ND
IT22	Random bred albino	Embryo (3T3)	47-70	<1	ND
Cl 1D	C3H/HeJ	Earle's L cells	210-213	125	16
L-F$_2$	C3H/HeJ	Earle's L cells		ND	16
MG57G	C57BL/6	Methylcholanthrene-induced tumor	310-325	255	ND
P815Y	DBA/2	Mastocytoma		128	32
L-5178Y	DBA/2	Lymphoma		64	32
F9	129/J	Embryo-derived teratocarcinoma		ND	<2
		SV40-Transformed cell lines			
C57SV	C57B1/6J	Embryo	160-170	60	8
SV3T3	BALB/c An	Embryo	90-110	722	16
16 lines	Various	Kidney	13-90	<1	≤2
K129SV	129/J	Kidney	93-96	1,200	32
KG^{-}SV	129 G$^{IX^{-}}$	Kidney	77-87	2,000	64
KT6SV	CBA/H-T6J	Kidney	75-80	125	64
K4RSV	B10.A(4R)	Kidney	70-77	600	32
4 lines	Various	Spleen	13-80	<1	≤2
5 lines	Various	Liver	21-30	<1	≤2
BT6SV	CBA/H-T6J	Blood	50-60	<1	1
BICR-TR	Random bred albino	BICR brain	6-8	<1	ND
		Adenovirus 5-transformed cell lines			
7 Lines	Various	Kidney	17-53	<1	<2

* ND = not done.

TABLE III

Induction of Anti-Viral Activity in Mixed Cultures Containing Mouse Lymphocytes and Xenogeneic, Allogeneic, or Syngeneic Cultured Cells

Cell line	Origin	Lymphocytes			
		H-2	BALB/c	C57BL/6	(BALB/c × B6)F$_1$
None	–	–	2*	<1	<1
RDMC	Human	–	32	8	8
SV3T3	BALB/c	d	16	4	8
P815	DBA/2	d	64	32	16
C57SV	C57BL/6J	b	16	4	4
K129SV	129/J	b	32	4	8
KT6SV	CBA/H-T6J	k	16	4	4

* Anti-viral units produced in the supernate of the mixed cultures.

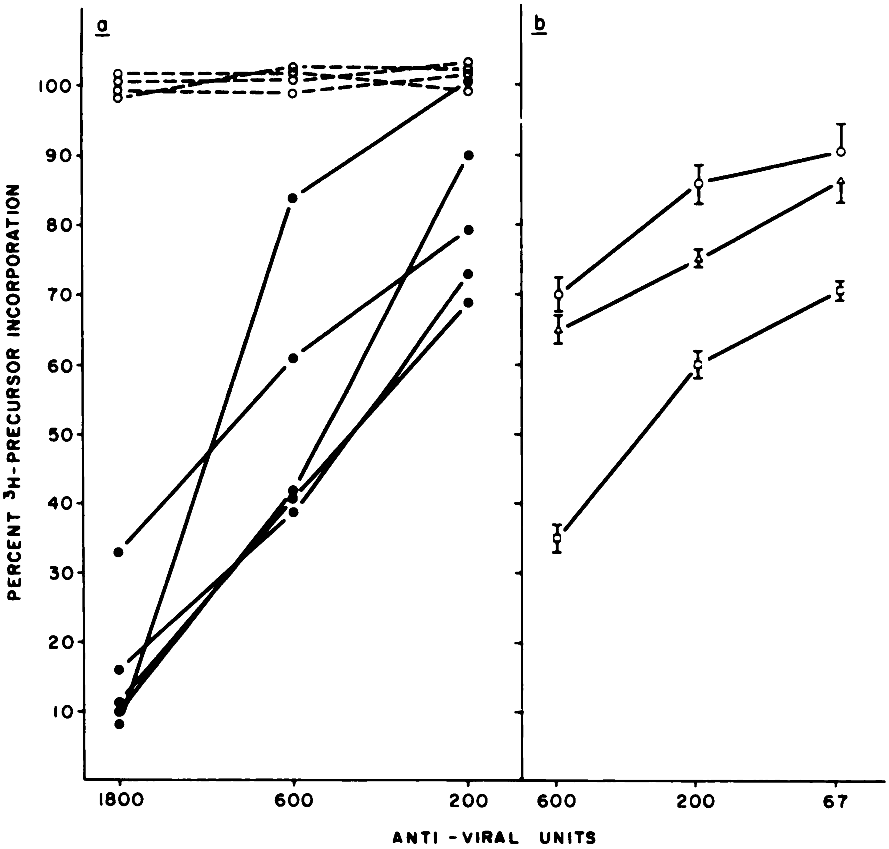

FIG. 1. Inhibition of thymidine, uridine, and leucine uptake in human and murine cells treated with supernates from mixed cultures of lymphocytes and RDMC cells. The experimental points are expressed as percent of the incorporation in control cultures. a:[^{3}H]thymidine incorporation in five different human cell lines (●——●) and four mouse cell lines (○——○). b: ○——○, [^{3}H]leucine uptake; ▽——▽, [^{3}H]uridine uptake; □——□, [^{3}H]thymidine uptake in RDMC cells. Vertical bars indicate standard deviation.

cell lines displayed an almost complete specificity for cells of the same species of the lymphocytes. Supernates from xenogeneic cultures of human lymphocytes mixed with murine cells or of mouse lymphocytes with human cells inhibited viral replication only in human and in mouse cells, respectively.

Characterization of the Viral Inhibitor. Supernate from human mixed cultures was not toxic for human fibroblast cells. Pretreatment for 18 h at 37°C of [^{51}Cr]labeled FS1 cells with a dilution of supernate from a mixed lymphocyte-RDMC culture containing 10^3 anti-viral U did not increase the spontaneous release of ^{51}Cr from the cells (36). The preparations containing anti-viral activity effectively inhibited viral replication at a dilution at which cellular DNA, RNA, and protein synthesis were not affected (Fig. 1).

The anti-viral activity in the supernate of mixed human lymphocyte human cell cultures was active on FS1 cells by using different challenge viruses (NDV, EMC, vaccinia virus, and influenza A (H3N2) virus).

The viral inhibitor in the supernate did not inhibit virus replication when

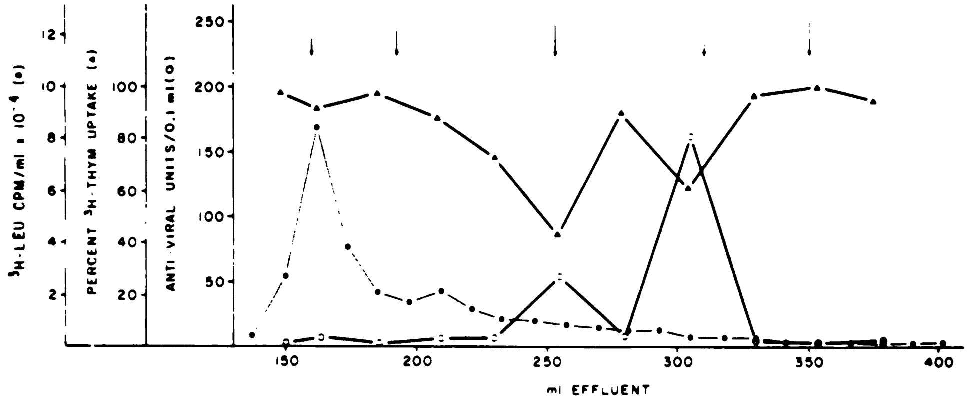

FIG. 2. Gel filtration on Sephadex G100 column of concentrated supernate from a mixed culture of human lymphocyte-RDMC cells. Cells were cultivated in the presence of 10 μCi [^{3}H]leucine/ml of medium. 30 ml of supernate was concentrated to 3 ml by vacuum dialysis and applied to the column (25 × 960 mm, 471 cm^3, void volume 160 ml). Arrows indicate molecular weight markers; from left to right: blue dextran 2,000 (mol wt 2,000,000), aldolase (158,000), ovalbumin (45,000), chymotrypsinogen A (25,000), ribonuclease A (13,700). The fractions were dialyzed against RMPI 1640, sterilized by filtration, and tested for anti-viral activity and ability to inhibit [^{3}H]thymidine uptake in RDMC cells.

added to the indicator cells at the same time as the virus; FS1 cells pretreated with the supernate and then carefully washed were unable to replicate VSV. Actinomycin D (1 μg/ml) or cycloheximide (250 μg/ml) present during treatment of FS1 with dilutions of supernates containing up to 200 anti-viral U completely prevented the inhibition of VSV replication.

The viral inhibitor was completely destroyed by treatment of diluted supernate with 0.1 mg/ml of trypsin (1 h incubation at 37°C). After gel filtration of concentrated supernate on a Sephadex G100 fine column, the anti-viral activity was recovered in two peaks of apparent mol wt of approximately 45,000 and 25,000 daltons (Fig. 2). In some experiments, a variable amount of activity was in front of the albumin peak. Chromatography of NDV-induced lymphocyte interferon (obtained after treatment at pH 2.0) gave only one peak of anti-viral activity with mol wt 25,000. When NDV interferon was not pretreated at pH 2.0, the same two peaks (45,000 and 25,000) were obtained. The anti-viral activity in the 25,000 peak was resistant to treatment at pH 2.0, whereas the activity in the 45,000 peak was destroyed under the same conditions.

The above characterization of the viral inhibitor in the supernates of mixed cultures allows their acceptance as interferons.

Characterization of the Human Lymphocyte Population Producing Interferon. Human lymphocytes, preincubated at 22°C for a period of up to 24 h, maintained the ability to produce interferon when induced by RDMC cells in mixed culture or by infection with NDV. In contrast, after 17 h of preincubation at 37°C, the lymphocytes were unable to be induced by RDMC cells, but still responded to NDV infection with an anti-viral activity reduced to about 20% of that observed with fresh cells.

The results of experiments with cell fractions of the lymphocyte population are presented in Table IV. Depletion of glass-adherent cells from Ficoll-Hy-

TABLE IV

Ability of Human Lymphocyte Subpopulations to Produce Interferon in Mixed Lymphocyte-RDMC Cultures

Fraction	ANAE[*]	S-Ig[‡]	E-RFC[§]	Fc-rec[‖]	Anti-viral units produced
	%	%	%	%	
Exp. A Ficoll-Hypaque separated	22.3	ND	ND	ND	125
Glass nonadherent	6.7	ND	ND	ND	3,000
Exp. B Ficoll-Hypaque separated	17.9	22.7	62.9	36.6	160
Nylon column nonadherent	1.4	2.4	86.4	20.2	640
Nylon column adherent	30.4	55.8	24.2	66.0	10
Exp. C Nylon column nonadherent	0.9	1.7	88.4	22.9	600
AET-SRBC Nonrosetting	11.4	32.0	0	48.5	3,000
AET-SRBC Rosetting	0	0	97.6	10.2	25
Exp. D Ficoll-Hypaque separated	23.6	14.2	77.2	38.2	80
IgG-Anti-IgG column nonadherent	9.3	0.9	89.4	9.8	<5
IgG Column adherent	21.0	14.9	73.1	29.4	40

[*] Staining for nonspecific acid esterase, strongly positive cells (monocytes) scored (32).

[‡] Positive fluorescence for surface immunoglobulin [fluorescein-rabbit $F(ab')_2$ anti-human $F(ab')_2$] (37).

[§] Cells forming rosettes with AET-treated sheep erythrocytes (18 h incubation at 4°C) (38).

[‖] Positive fluorescence for Fc-receptor with heat aggregated human IgG (39) and fluroescein-labeled anti-human IgG.

ND, not done.

SRBC, sheep erythrocytes.

paque-separated lymphocytes increased the level of anti-viral activity obtained by induction with RDMC cells (Exp A). The major population responsible for interferon production in mixed cultures of lymphocytes and RDMC cells was not adherent to nylon fiber columns, did not form rosettes with AET-treated sheep erythrocytes, and was retained on an Ig-anti-IgG column. The cell fractions in Exp. C of Table IV were all incubated with erythrocytes and subjected to hypo-osmotic treatment to avoid artifactual results due to different handling of the subpopulations.

Anti-Cellular Activity. Supernates from mixed cultures of human lymphocytes and cells from RDMC or any other interferon-inducing cell line inhibited [^{3}H]thymidine incorporation in human cells from several lines, but not in cells from murine lines (Fig. 1a). [^{3}H]leucine and [^{3}H]uridine incorporation were also inhibited in human cells, but not to as great an extent as was [^{3}H]thymidine incorporation. The supernates from unmixed cultures of lymphocytes or of cells from the various lines tested and the supernates from mixed cultures of lymphocytes and cell lines unable to induce interferon did not inhibit incorporation. The activity inhibiting thymidine incorporation and the anti-viral activity were significantly correlated in 15 supernates from mixed cultures of lymphocytes from different donors and RDMC cells ($r = 0.865, P < 0.001$, Fig. 5). About 500 anti-viral U were required to inhibit 50% uptake in RDMC cells (Figs. 1b and 3). In gel filtration experiments the activity inhibiting thymidine incorporation was eluted in the same two peaks of the anti-viral activity.

Inhibition of Cell Growth. Supernates from mixed cultures of lymphocytes and cells from RDMC or other interferon-inducing lines were able to inhibit cell growth in different human cell lines (Fig. 4). This effect was detected only when supernate was used in concentrations containing more than 500 anti-viral U. The supernate from lymphocytes cultured alone or from RDMC cells cultured alone had no significant effect (Fig. 4).

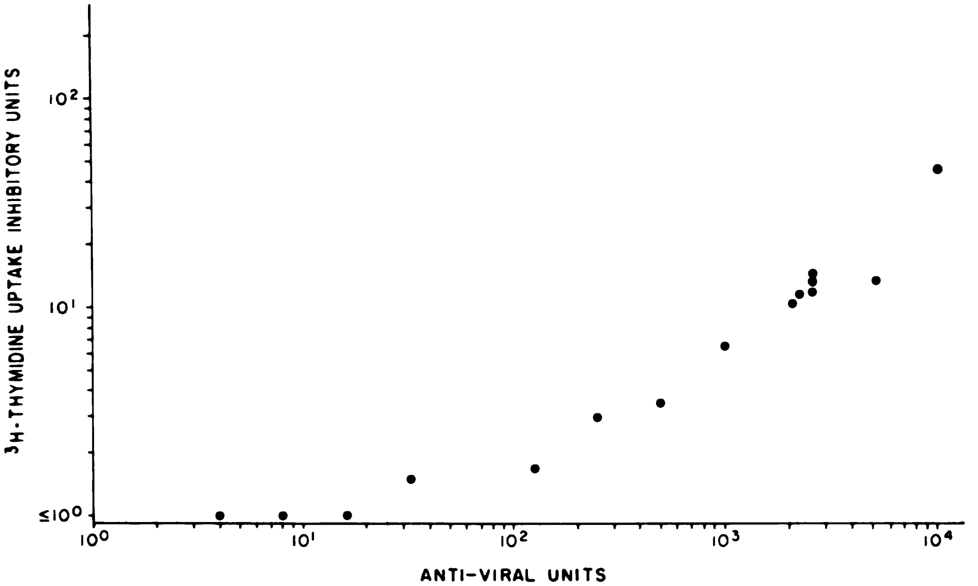

FIG. 3. Anti-viral and anti-cellular (inhibition of thymidine uptake) activity in 15 supernates from mixed cultures of RDMC cells and human lymphocytes from different donors. Each dot represents the results obtained with one interferon preparation.

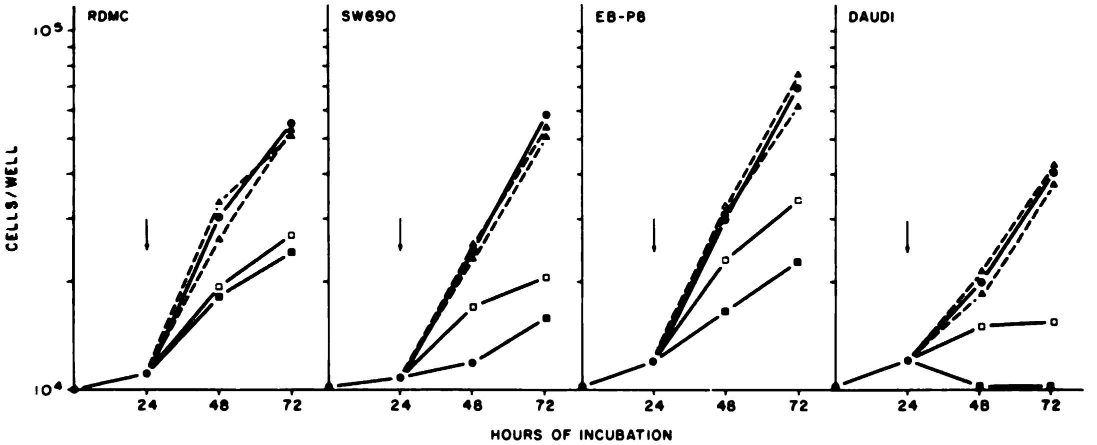

FIG. 4. Inhibition of cell growth of four different cell lines (RDMC, SW690, EB-P8, and Daudi) by supernate from mixed lymphocyte-RDMC cultures. The arrows indicate the times when supernates (0.1 ml) were added to cells (incubated in 0.1 ml medium). ●——●, medium only; ■——■, mixed lymphocyte-RDMC supernate, 1,800 anti-viral U; □——□, 600 anti-viral U; △——△, lymphocyte supernate; ▲——▲, RDMC supernate.

Discussion

A virus inhibitor(s) is released in the supernate of mixed cultures of lymphocytes and certain tumor-derived and virus-transformed cell lines after a few hours of incubation (23). The species preference of the inhibitor produced in the xenogeneic mixed cultures identifies the lymphocytes as the producers of the inhibitor. The viral inhibitor(s) from human mixed cultures meets the criteria established by Lockart (40) for acceptance of a viral inhibitor as an interferon: (*a*) it is a protein that is readily destroyed by incubation with trypsin; (*b*) it does not inhibit viral replication through a toxic effect on the cells; (*c*) it is active against all viruses tested; (*d*) it does not directly inactivate the virus; instead it induces an anti-viral status in the cells that persists when the inhibitor is removed from the medium; synthesis by the cells of both RNA and protein are required for induction of the anti-viral status. In addition, the viral inhibitor shows other properties that are common to most interferons: it is more active in homologous than heterologous cells; it is not released spontaneously but only in the presence of the inducer cell line; one of the two molecular populations identified by gel filtration is stable at pH 2.0.

The gel filtration experiments show that at least two molecular species of interferon are present in the supernate of the mixed cultures. The viral inhibitor which elutes in a peak corresponding to a mol wt approximately 25,000 represents the majority of the activity. This interferon is stable at pH 2.0, and is probably corresponding to the type I interferon of Youngner and Salvin (41). Type I interferon in the mouse is produced by cells other than T cells and can be induced by viruses and by B-cell stimulants (41, 42). In humans, NDV-induced leukocyte interferon (43, and our results) also has a mol wt of approximately 25,000. The viral inhibitor in the supernate of mixed cultures, which is eluted with a peak of approximately 45,000, is unstable at pH 2.0 and shares most of the characteristics of the interferon designed as type II (41) or immune (44) interferon. In the mouse, this type of interferon is considered to be a product of T cells activated mainly by antigenic or mitogenic stimulation (42, 44). However, we have found some anti-viral activity in a peak corresponding to a mol wt of 45,000 after gel filtration of NDV-induced interferon that had not been pretreated at pH 2.0. We have also found the presence of a variable amount of antiviral activity eluting in front of the albumin peak possibly due to molecules of interferons that bind to albumin or other proteins (45).

Cell separation experiments have been performed to identify the population of human peripheral blood lymphocytes necessary for interferon production in the mixed cultures. Monocytes are not only unnecessary for interferon production in mixed cultures but also have an inhibitory effect. Depletion of B cells (surface immunoglobulin-bearing cells) and monocytes by incubation on nylon fiber columns increased interferon production by the eluted cell population. The B-cell-enriched populations recovered from the columns produced very little interferon. On the other hand, lymphocytes depleted of both surface immunoglobulin-bearing cells and Fc-receptor-bearing cells (on IgG-anti-IgG columns) were capable of producing only minimal amounts of interferon in the mixed culture experiments. These data suggest that the major effector cell population is composed of lymphocytes without surface immunoglobulins but with Fc-receptors. To evaluate the role of T cells in the production of interferon we employed AET-treated sheep erythrocytes, reported to be the most sensitive for rosetting techniques (38). When corrected for the different numbers of cells recovered in the two populations (usually 4–10 times more cells in the rosetting fraction), the data indicate that most of the interferon-producing lymphocytes do not bind to AET-treated sheep erythrocytes. These results exclude a major role of T cells in the production of interferon in this system. All the lymphocyte subpopulations able to produce interferon also contained most of the activity of the natural killer cells. Natural killer cells not only spontaneously interact with and lyse inducer cells in the mixed cultures (46), thus possibly playing some role in the induction of interferon itself, but their cytotoxic activity is also strongly enhanced by the presence of interferon in the culture (36).

In addition to the anti-viral activity, an anti-cellular activity is present in the supernate of mixed human lymphocyte human cell cultures. The major effect on cellular metabolism is inhibition of DNA synthesis, as measured by inhibition of [^{3}H]thymidine uptake. That this assay actually measures inhibition of DNA synthesis and not an artifactual inhibition of the uptake of the labeled precursor, as observed with supernate from macrophage and lymphocyte cultures (47, 48), is suggested by these facts: the inhibitory activity is not dialyzable and it inhibits cell proliferation. Although a close correlation between anti-cellular and anti-viral activity has been observed in the present experiments, as in several other studies with virus-induced interferon (49–52), no conclusion can be derived on the unsettled issue of whether or not the anti-cellular and the anti-viral activities in interferon preparations are mediated by the same molecules (45, 49–53).

The characteristics that make certain cell lines able to induce interferon and the nature of the stimulus that triggers the lymphocytes are still unknown (23). Although the association of ability to induce interferon with transformation by EBV or mouse sarcoma virus or with the tumor origin of the line is statistically significant, any speculation on the biological relevance of such an association seems premature. That stimulation by allogeneic (or heterologous) antigens are responsible for stimulation may be excluded by the induction of interferon in autologous or syngeneic mixed cultures, both in human and in the mouse.

The possibility that viruses present in the cell lines are directly responsible for the induction seems to be unlikely. The possibility remains that some viruses usually associated with inducer cell lines (murine sarcoma virus, EBV), unknown viruses or other agents derived from the original tumor or acquired in culture induce particular antigenic or structural surface characteristics that are responsible for the interaction with the lymphocytes and their stimulation.

It is not possible to extrapolate the data obtained in vitro with cell lines to infer a stimulation of interferon in vivo by spontaneous tumors. Indeed, a hyporeactive factor, which inhibits interferon production, is present in the serum of tumor-bearing mice (54, 55). This factor could mask a stimulation by tumor cells. However, at least in the case of transplantable tumors in mice, our experiments and a preliminary report by others (56) show interferon induction by syngeneic tumor cells in vivo: because of the effect of interferon both on the host defense mechanisms and on the tumor cell metabolism (2) this phenomenon might affect the progress of the transplanted tumor.

Summary

A viral inhibitor(s) is released in the supernate of mixed cultures containing human or mouse lymphocytes and cells from certain lines. The inhibitor is active against a variety of unrelated viruses and is a protein that is not toxic for cells. It does not inactivate viruses directly, but inhibits viral replication through an intracellular mechanism that involves synthesis by the cells of both

RNA and protein. These characteristics identify the inhibitor as an interferon. The anti-viral activity is contained in at least two molecular species, of approximately 25,000 and 45,000 daltons, respectively. In addition to the antiviral activity, the supernates of the mixed cultures display an anti-cellular activity, the inhibition of DNA synthesis and of cell multiplication. The antiviral and the anti-cellular activities are positively correlated in supernates from various cultures and in partially purified preparations. The human cell population responsible for interferon production is composed mainly of Fc-receptor positive, surface immunoglobulin negative, non-T-cell lymphocytes. The ability of certain cell lines to induce interferon seems to be preferentially associated with tumor origin or with in vitro transformation by certain viruses (Epstein-Barr virus, murine sarcoma virus).

We gratefully acknowledge Doctors Walter Gerhard, Meenhard Herlyn, Kay Huebner, Albert Leibovitz, Jon Palmer, Zenon Steplewski, Roberto Weinmann, and Tadeusz Wiktor for providing us with viruses and cell lines, Dr. Elliot Levine for performing the mycoplasma tests, and Ms. Katherine M. Maloney, Patricia Radich, Nancy Seitz, and Lucy Sheffield for their excellent technical assistance.

Received for publication 13 October 1977.

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ANTI-VIRAL ACTIVITY INDUCED BY CULTURING LYMPHOCYTES WITH TUMOR-DERIVED OR VIRUS-TRANSFORMED CELLS

Enhancement of Human Natural Killer Cell Activity by Interferon and Antagonistic Inhibition of Susceptibility of Target Cells to Lysis*

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Accumulating evidence indicates that interferons may have a regulatory role in several immune functions. Interferons suppress proliferation of lymphocytes in response to mitogens and antigens (1), inhibit antibody formation (2–4), and enhance the generation of specific cytotoxic effector cells (5, 6). The phagocytic activity of mouse macrophages (7, 8) and the suppression of tumor cell growth mediated by mouse spleen cells (9) have been shown to be enhanced by interferon in in vivo and in vitro experiments.

Lymphocytes from human peripheral blood obtained from any normal donor are spontaneously cytotoxic in vitro for target cell lines (10–14). The cytotoxic activity is mediated by natural killer cells that have been identified as Fc-receptor positive, surface immunoglobulin negative lymphocytes (11, 12). Only a small proportion of natural killer cells form rosettes with sheep erythrocytes (11, 12, 15, 16) when very sensitive techniques employing erythrocytes treated with a sulphydryl reagent (17) or with enzymes (18) are used. The natural killer cells in human peripheral blood cannot be separated from cells involved in antibody-dependent cell-mediated lysis which suggests that the two types of cytotoxicity may be mediated by the same cell population (12–14, 19).

We have shown that cells from certain tumor-derived or virus-transformed lines effectively induce interferon production when cultured together with human or mouse lymphocytes (20). For the present study we investigated the effects that the interferon produced in such mixed cultures exerts on the two cell populations: interferon enhances the spontaneous cytotoxic activity of the lymphocytes and has an antagonistic inhibitory activity on the susceptibility of the target cell lines to cell-mediated lysis.

Materials and Methods

Reagents. Cycloheximide and 2-aminoethylisothio-uronium bromide hydrobromide (AET)[1]

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* Supported in part by U. S. Public Health Service research grant CA20833, CA10815, CA43882 from the National Cancer Institute, NS11036 from the National Institute of Neurologic and Communicative Diseases and Strokes, by grant IM-88 from the American Cancer Society, and by the national Multiple Sclerosis Society.

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[1] *Abbreviations used in this paper:* AET: 2-aminoethylisothio-uronium bromide hydrobromide; FBS, fetal bovine serum; TCID_{50}, tissue culture infectious dose 50%; HAU, hemagglutination unit; NDV, Newcastle disease virus; PBS, phosphate-buffered saline; PFU, plaque-forming units; PHA, phytohaemagglutinin; RDMC, rhabdomyosarcoma cell line; VSV, vesicular stomatitis virus; BCG, Bacillus Calmette-Guérin.

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were obtained from Sigma Chemical Co. (St. Louis, Mo.); actinomycin D from Calbiochem (San Diego, Calif.); fluorescein isothiocianate-conjugated goat $F(ab')_2$ fragment anti-human IgG $F(ab')_2$ fragment from Cappel Laboratories, Inc. (Cochranville, Pa.); and sheep erythrocytes from Flow Laboratories, Inc. (Rockville, MD).

Cell Lines and Viruses. The origins of most of the cell lines used in this study are summarized in Table I (20). Adherent cells were subcultured in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (FBS) (Flow Laboratories). The human lymphoid line EB-P8 was obtained by transforming peripheral blood lymphocytes from a multiple sclerosis patient with Epstein-Barr virus. It was subcultured in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 international U/ml), and streptomycin (100 μg/ml) (RPMI-FBS, Flow Laboratories).

Newcastle disease virus (NDV) was obtained from T. J. Wiktor (Wistar Institute) [allantoic fluid, 3.3×10^9 plaque-forming units (PFU)/ml on L929 mouse cells]. The Indiana strain of vesicular stomatitis virus (VSV) was originally obtained from T. J. Wiktor and grown in tissue culture on human rhabdomyosarcoma-derived cells (RDMC) (3×10^8 PFU/ml on L-F2 mouse cells). Vaccinia virus (Lister strain), provided by M. Herlyn (Wistar Institute) was originally obtained from Wyeth Laboratories (Radnor, Pa.) and grown in WI38 cells [10^6 tissue culture infectious doses 50% ($TCID_{50}$)/ml on WI38 cells]. The Hong Kong/107/68 (H3N2) strain of influenza A virus was obtained from W. Gerhard (Wistar Institute) (allantoic fluid, 850 hemagglutination units [HAU]/ml).

Lymphocyte Separation and Culture. Mononuclear cells were obtained from heparinized human peripheral blood by separation on a Ficoll-Hypaque gradient. The methods for separating subpopulations of human lymphocytes have been previously described (20). Briefly, lymphocytes were depleted of monocytes and of most B cells by incubation on a nylon column, according to a modification (20) of the method of Julius et al. (21). T-cell-enriched and depleted fractions were obtained by separating lymphocytes rosetting with AET-treated sheep erythrocytes (22) from nonrosetting lymphocytes on a Ficoll-Hypaque gradient. Erythrocytes were eliminated from rosetted cells by distilled water lysis. For culturing lymphocyte preparations in the presence or absence of interferon, cell suspensions at 5×10^6 cells/ml in RPMI-FBS were incubated at 37°C in a humidified 5% CO_2 atmosphere, in standing tissue culture flasks (3013, 2–3 ml/flask; 3024, 10 ml/flask; BioQuest, BBL, & Falcon Products, Becton, Dickinson & Co., Cockeysville, Md.). At the end of the incubation, cells were washed three times in phosphate-buffered saline (PBS), pH 7.2, and viable cells were counted with an erythrosin B solution (23) and resuspnded in RPMI-FBS.

NDV Induction of Lymphocyte Interferon. NDV at a multiplicity of infection (MOI) of 20 was added to a suspension (10^7 cells/ml in RPMI-FBS) of human lymphocytes. After an 18 h incubation (37°C) the cells were eliminated by centrifugation (30 min, 30,000 *g*); the supernatant was then brought to pH 2.0 with HCl, 1 N, incubated for 5 days at 4°C, neutralized to pH 7.0 with NaOH 1 N, passed through a sterile filter (porosity 0.45 μm, Nalge Co., Nalgene Labware Div., Rochester, N.Y.) and stored at −80°C.

Interferon Production in Mixed Tumor-Lymphocyte Cultures. Human lymphocytes were depleted of adherent cells by two passages (1 h each at 37°C) on glass Petri dishes and resuspended at 10^7 cells/ml in RPMI-FBS. 8–10 ml of the cell suspension was added to confluent monolayers of RDMC cells in 75 cm^2 tissue culture flasks (3024, BioQuest, BBL, & Falcon Products) and incubated 18 h at 37°C. The supernates were centrifuged at 30,000 *g* for 30 min, filtered through a sterile 0.45 μm filter, and stored at −80°C.

Interferon Assay. Anti-viral titers of human interferon preparations were measured as previously described (20) by inhibition of the cytopathic effect of VSV on a monolayer of human fetal skin fibroblasts (FS1). Anti-viral units are expressed as the reciprocal of highest dilution inhibiting 50% of the cytopathic effect and are equivalent to approximately 1 reference U of the NIH Human Reference Interferon G-023-901-527.

Cell-Mediated Cytotoxicity on Adherent Target Cells. Trypsinized target cells were seeded into the wells of flat bottom microtiter plates (3040, BioQuest, BBL, & Falcon Products) at a dose of 2×10^4 cells/well; 2 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear Corp., Boston, Mass.) were added to each well, and the plates were incubated overnight at 37°C. The plates were then washed three times and 0.1 ml of RPMI-FBS or interferon diluted in medium was added in each well. Effector cell dilutions of 0.1 ml were added, and the plates were incubated at 37°C in a humidified 5% CO_2 atmosphere for the indicated times. After centrifugation, 0.1 ml of the supernatant A was then collected from each well and 0.1 ml of 1% Triton X-100 added. The plates were further incubated for at least 4 h at 37°C and 0.1 ml of supernatant B was again collected from each well. Samples A and B were counted for ^{51}Cr activity; percent ^{51}Cr release (E) was calculated as:

$$E = \frac{A}{B + A/2} \times 100.$$

All experimental and control values were the mean of at least three replicates. Where S is the percent ^{51}Cr release from target cells in the presence of medium alone, the specific percent ^{51}Cr release (R) was computed as:

$$R = \frac{E - S \times 100}{100 - s}.$$

Antibody-Dependent Cell-Mediated Cytotoxicity. The method has been previously described in detail (13, 24, 25). The established lymphoblastoid B-cell line EB-P8, with phenotype HLA-A3, B5, 7 was used as target. [^{51}Cr]labeled target cells were sensitized with a mixture of anti-HLA sera BsSh (anti-B7, diluted 1/20) and ToBU 1.64 (anti-B5, 12, purified IgG, 0.1 mg/ml) both of which were obtained from the Instituto di Genetica Medica, University of Torino (Italy).

Computation of Lytic Units. Dose-response curves of specific cytotoxicity (both for antibody-dependent and direct cell-mediated cytotoxicity) were determined by plotting specific ^{51}Cr release versus the number of effector lymphocytes. The number of lymphocytes necessary to lyse 50% of the target cells in the incubation time was referred to as 1 lytic U. Determination of the 50% lytic units was done graphically or computed on the basis of a modification (24) of the van Krogh equation.

Mouse Anti-Human Cytotoxic T Cells. A cell-mediated cytotoxic response against human cells was elicited in C57BL/6J mice by intraperitoneal injection of 3 × 10^7 RDMC cells, as previously described (26). The cytotoxic test, using effector spleen cells on human adherent target cells, was performed as described above, at the peak of the cytotoxic response 11 days after immunization.

Results

Additions of Exogenous Interferon to Lymphocyte-Target Cell Cultures. Interferon preparations induced in human lymphocytes either by culturing with RDMC monolayers or by exposure to NDV were added to a mixture of lymphocytes and target RDMC cells in an assay of cell-mediated cytotoxicity. In the cultures in which interferon was added an enhancement of cytotoxicity, usually from two to fourfold, was observed at 4–8 h of incubation (Fig. 1). The rate of cytotoxicity after 8 h of incubation was usually not higher in cultures to which interferon had been added than in control cultures, high levels of endogenous interferon being present in both cultures (Fig. 1). Some cell lines were unable to induce production of interferon from lymphocytes (Table I); the use of these cells as targets allowed us to detect, in some experiments, an interferon-induced increase in the rate of cytotoxicity at longer incubation times also. However, the antagonistic effect of interferon on the susceptibility to lysis of most target cells (see below), often reduced the observed effect of exogenous interferon preparations on cytotoxicity.

Effect of Interferon Produced in the Lymphocyte-Target Mixture on Cell-Mediated Cytotoxicity. Lymphocytes incubated at 37°C for 24 h completely lose their ability to produce interferon when cultured with cells from inducer lines (20). This observation allowed us to evaluate indirectly the effect of interferon produced in mixed cultures on the spontaneous cell-mediated cytotoxicity of human lymphocytes. Lymphocytes that were freshly separated or previously incubated 24 h at 37°C were tested as effector cells in an 18-h cytotoxic test (Fig. 2). No difference was observed in the cytotoxic efficiency of fresh versus preincubated lymphocytes when cells were tested against targets unable to

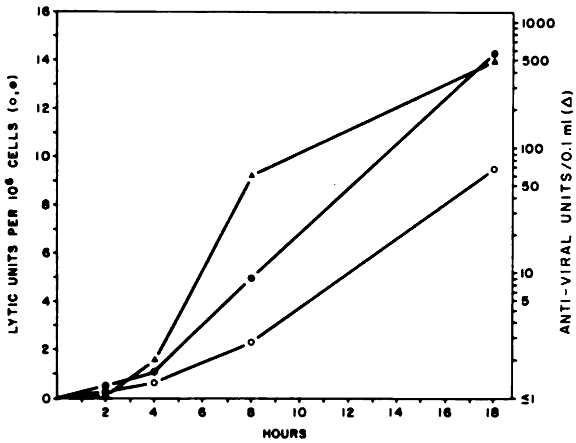

FIG. 1 Effect of exogenous interferon on spontaneous cytotoxicity mediated by human lymphocytes against RDMC target cells. ●——●, 500 anti-viral U of RDMC-induced lymphocyte interferon added to lymphocyte-target cell mixtures at the beginning of the cytotoxic test; ○——○, no exogenous interferon added during the cytotoxic test; △——△, endogenous anti-viral activity in the supernate of the cultures with no exogenous interferon. At 4, 8, and 18 h of incubation the amounts of ^{51}Cr release were significantly different ($P < 0.01$, Student's *t* test) between cultures to which interferon was added and those to which it was not (considering only the dilutions of effector lymphocytes giving cytotoxic values in the linear part of the titration curve, 30–70%).

induce interferon (Fig. 2b). In contrast, when interferon-inducing cells were targets, the cytotoxicity of the preincubated lymphocytes was reproducibly decreased to only 10–30% of that observed with freshly separated cells (Fig. 2a). This observation, reproducible when cell lines other than those in Fig. 2 were used as targets, suggests that the interferon released in the culture mediates an enhancement of cytotoxicity that represents up to 70–90% of the total spontaneous cell-mediated cytotoxicity observed with fresh lymphocytes in an 18 h test.

Pretreatment of Lymphocytes with Interferon. When lymphocytes were pretreated for 18 h with interferon preparations then washed and tested as effector cells against several different target cell lines, they displayed a cytotoxic efficiency up to 10-fold higher than that of untreated lymphocytes (Fig. 3). The increase in the nonspecific cytotoxicity of human lymphocytes cultured in the presence of interferon is quantitatively comparable to or higher than that observed upon stimulation of lymphocytes with optimal concentrations of phytohaemagglutinin (PHA) (Fig. 3). In some experiments interferon-treated cytotoxic lymphocytes induced more than a 50% ^{51}Cr release in a 4-h test (RDMC target) at a ratio of effector to target of 1:1. These levels of cytotoxicity were maintained or increased after lymphocytes were treated for 48 h with interferon; beginning on the 3rd day cytotoxicity declined to levels close to those of untreated lymphocytes (Fig. 2). At 5–7 days in culture, a spontaneous increase in the cytotoxicity of the lymphocytes was usually observed: the presence of interferon in the culture depressed this late increase (Fig. 2).

The enhancement of cytotoxicity was dependent on the dose of interferon added to the culture; a twofold increase was obtained with about 10 anti-viral U

TABLE I

Human Cell Lines: Ability to Induce Interferon When Cultured with Lymphocytes and Susceptibility to the Interferon-Induced Resistance to Cytotoxic Lymphocytes and Inhibition of Viral Replication

Cell line	Origin	Passage	Units Interferon Induced* (mean ± Standard Error)	Inhibition of cytotoxicity‡	Inhibition of viral replication§
				%	
FS1	Fetal skin fibroblasts	14	<1	92	4.0
Pa	Skin fibroblasts	8	<1	86	3.6
LR-1	Newborn brain	4	<1	82	3.3
WI38	Fetal lung fibroblasts	42	<1	62	3.0
MRC5	Fetal lung fibroblasts	45	<1	40	1.8
LN-SV	Skin, SV40-transformed	253	<1	77	3.0
W18.VA2	Lung, SV40-transformed	245	<1	75	2.1
S1054TR	Brain, SV40-transformed	32	193 ± 137	20	<0.3
SW690	Melanoma	90	850 ± 552	75	2.4
SW691	Melanoma	85	6,000 ± 3,000	13	<0.3
SW480	Colorectal carcinoma	112	125 ± 0	24	<0.3
D98 (HeLa)	Cervical carcinoma	>60	312 ± 165	30	<0.3
HT1080	Fibrosarcoma	117	0	15	0.6
RDMC	Rhabdomyosarcoma	203	3,494 ± 1,008	18	<0.3

* Anti-viral units in the supernate (24 h incubation of mixed cultures of monolayer of the cell line with human lymphocytes; four or more different lymphocyte preparations tested for each cell line).

‡ Average percent inhibition of cytotoxicity. After treatment with interferon (18 h, 10^3 anti-viral U), cells from the various lines were tested as target against two different preparations of effector lymphocytes (stimulated by 10^3 anti-viral U of NDV-induced lymphocyte interferon, 18 h).

§ Log_{10} of the reciprocal of the dilution of a lymphocyte interferon preparation (NDV-induced, 10^4 anti-viral reference U) inhibiting 50% of the cytopathic effect of VSV or monolayer of the cell line.

of interferon administered for 18 h (Fig. 4 and 5b). The same enhancement of cytotoxicity was obtained, at equivalent anti-viral doses, with interferon preparations obtained by stimulation of human lymphocytes with either virus (NDV) or an inducer cell line (RDMC). When the anti-viral activity and the cytotoxicity-enhancing activity of 14 RDMC-induced interferon preparations from various lymphocyte donors were compared, a significant positive correlation was observed ($P < 0.01$, Fig. 5b). No enhancement of cytotoxicity was obtained with supernate from lymphocytes or inducer cell lines cultured alone. Supernates from mixed cultures of allogeneic lymphocytes (24 h incubation) and from cultures of lymphocytes with noninducing cell lines neither displayed activity nor enhanced cytotoxicity.

The cytotoxicity-enhancing activity in the human interferon preparation was destroyed by trypsin treatment (1 mg/ml, 1 h at 37°C, neutralized with soybean trypsin inhibitor bound to agarose beads) and was resistant to 5 days treatment at pH 2.0. The cytotoxicity-enhancing activity in the RDMC-induced preparations was eluted from a Sephadex G100 column in a peak of approximate mol wt

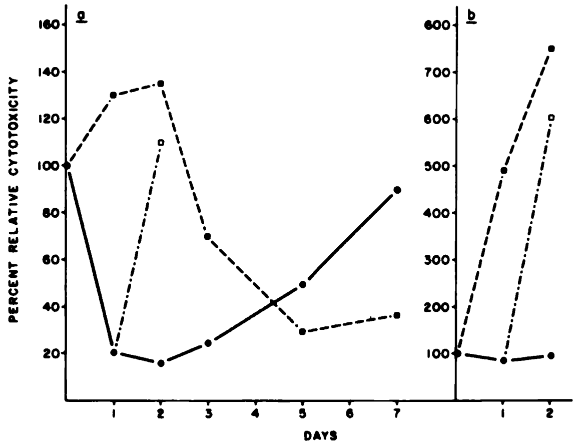

FIG. 2. Effect of interferon on the cytotoxic efficiency of human lymphocytes in culture. The lymphocytes were cultured for the number of days indicated in the presence or absence of 10^3 anti-viral U of RDMC-induced interferon. Their cytotoxic activity was then tested in an 18-h test against an interferon-inducing target, RDMC (Fig. 2a), and against a noninducing target, FS1 (Fig. 2b). The relative cytotoxicity is expressed as the ratio of the lytic units of the cultured lymphocytes over the lytic units of the freshly separated ones. ●——●, no interferon added; ■-----■, interferon added at time 0; □-----□, interferon added after 24 h of culture at 37°C.

of 25,000 daltons, corresponding to the major peak of anti-viral activity (Fig. 6a). Some activity was observed in correspondence with the minor peak of anti-viral activity of approximate mol wt 45,000 (20). A variable amount of anti-viral activity and concordantly of cytotoxicity-enhancing activity, were also eluted in different experiments with the major peak of protein close to the front of the column; these results are presumably due to the ability of the interferon molecules to bind to albumin and other proteins (27).

Medium from lymphocytes stimulated with PHA (HA15, Wellcome Research Lab., Beckenham, England, final dilution 1:100, 24 h incubation at 37°C) contained some anti-viral (5–50 anti-viral U) and was able to enhance lymphocyte cytotoxicity. After gel filtration of the supernate from PHA-stimulated lymphocytes both the anti-viral activity and the cytoxicity-enhancing activity were recovered in a peak discrete from the higher molecular weight activity associated with the lectin molecules.

Mouse interferon preparations obtained from spleen cells and from L cells were almost ineffective in enhancing human lymphocyte cytotoxicity.

Characterization of the Cytotoxic Lymphocytes Affected by Interferon. The cytotoxic lymphocytes showing activity enhanced by interferon were characterized by two techniques: adherence to a nylon fiber column and gradient separation of cells rosetted with AET-treated sheep erythrocytes. With these separation methods the cytotoxic activities of spontaneous (natural) killer cells, antibody-dependent killer cells and the cytotoxic cells with activity enhanced by interferon were always associated with the same fractions (Table III). The effector cells responsible for the three activities were non-phagocytic, did not adhere to nylon fiber columns, had no detectable surface immunoglobulins, and

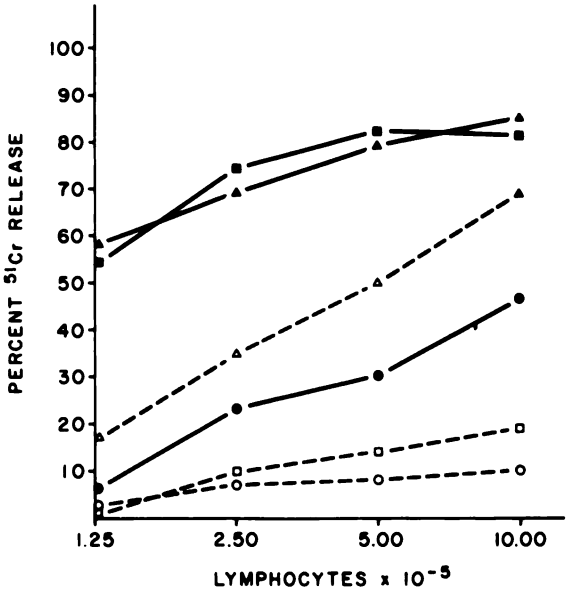

FIG. 3. Effect on cell-mediated cytotoxicity of pretreatment of lymphocytes and target cells with interferon preparations. Human lymphocytes were culture for 18 h at 37°C in the presence of 10^3 anti-viral units of RDMC-induced interferon, PHA (HA15 Wellcome, final dilution 1/100) or normal RPMI-FBS medium, washed three times, and then tested as effector cells (18-h test) on FS1 target cells pretreated or not pretreated with 10^3 anti-viral U of interferon. The SD of each experimental point was less than 2%. ●——●, untreated lymphocytes, untreated target cells; ○-----○, untreated lymphocytes, interferon-treated target cells; ■——■, interferon-treated lymphocytes, untreated target cells; □-----□, interferon-treated lymphocytes, interferon-treated target cells; ▲——▲, PHA-stimulated lymphocytes, untreated target cells; △-----△, PHA-stimulated lymphocytes, interferon-treated cells.

exhibited very low affinity for sheep erythrocytes (even when the extremely sensitive rosetting technique with AET-treated sheep erythrocytes was used) (28) (Table II).

When the effect of interferon pretreatment on the antibody-dependent cytotoxic activity of human lymphocytes was tested, no significant increase of the antibody-dependent activity was observed in repeated experiments although interferon strongly increased the cytotoxicity of the pretreated lymphocytes against the target lymphoid line in the absence of anti-target antibodies (Table III).

Inhibition of Target Cell Susceptibility to Lysis by Pretreatment with Interferon. Incubation of FS1 cells with 10^3 anti-viral U of RDMC-induced interferon for 18 h had no toxic effects. The spontaneous release of ^{51}Cr in 18 h was 27.8 ± 2.1 (mean ± SD, six experiments) from untreated target cells and 28.3 ± 3.3 from pretreated cells: the difference was not significant (Student's test for paired data, $t = 0.42$, NS). The preincubation of FS1 target cells with interferon induced up to 98% protection against the cytotoxicity mediated either by fresh or by interferon-stimulated human lymphocytes (Fig. 3). To reach the maximal level of inhibition it was necessary to preincubate the target cells for several

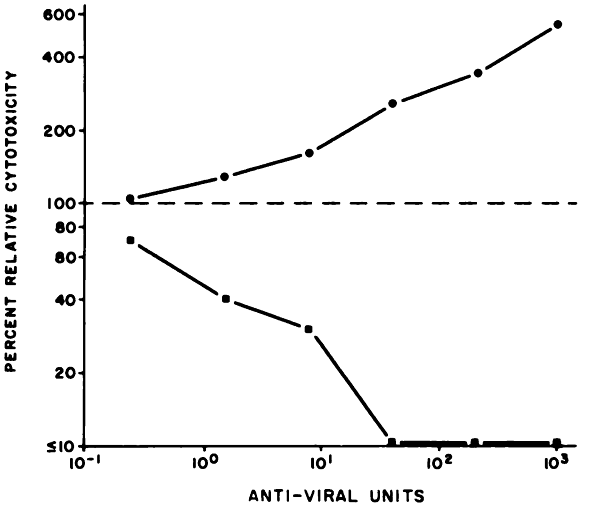

Fig. 4. Effect on lymphocytes and FS1 target cells of pretreatment with different doses of interferon. Lymphocytes, ●-----●, and FS1 target cells, ■——■, were incubated for 18 h in the presence of the indicated anti-viral units of RDMC-induced interferon and then tested reciprocally against untreated FS1 target cells or against lymphocytes pretreated with 10^3 anti-viral U of interferon. The relative cytotoxicity is expressed as the ratio between lytic units observed with interferon-pretreated and not pretreated lymphocytes or target cells.

hours (Fig. 7). The presence of interferon was not required for the entire period. Treating of FS1 cells for 30 min with interferon, then incubating them for 24 h without interferon induced an 85% inhibition of cytotoxicity, whereas pretreatment for 30 min immediately before the test induced only 35% inhibition (Fig. 7). The induction of resistance to lysis upon pretreatment with interferon (Fig. 8a) was prevented by concurrent treatment of FS1 cells with an inhibitor of RNA synthesis (actinomycin D, Fig. 8b) or with an inhibitor of protein synthesis (cycloheximide, Fig. 8c). Virus infection of the target cells (influenza A and vaccinia viruses) also prevented the induction of most of the resistance in FS1 cells (Fig. 8d and e).

14 different cell lines have been tested for interferon-mediated induction of resistance to the cytotoxic effect of lymphocytes (Table I). Fibroblasts are susceptible to both the anti-viral and target protective activities of interferon. The fetal lung fibroblasts WI38 and MRC5, tested in the experiment shown in Table I, were at very high passage and were relatively less susceptible to both interferon activities; other experiments with lower passages of these same cells showed a higher susceptibility to the two activities. When various SV40-transfomed or tumor-derived cell lines were tested, a variable susceptibility to interferon was observed in different lines. The tumor-derived lines tested, however, were not susceptible to either the anti-viral or the target protective activity of interferon, with the exception of the melanoma-derived SW690 cell line. A significant positive correlation was observed between cell line susceptibility to the anti-viral and to the target protective activity of interferon (r = 0.954, $P < 0.001$).

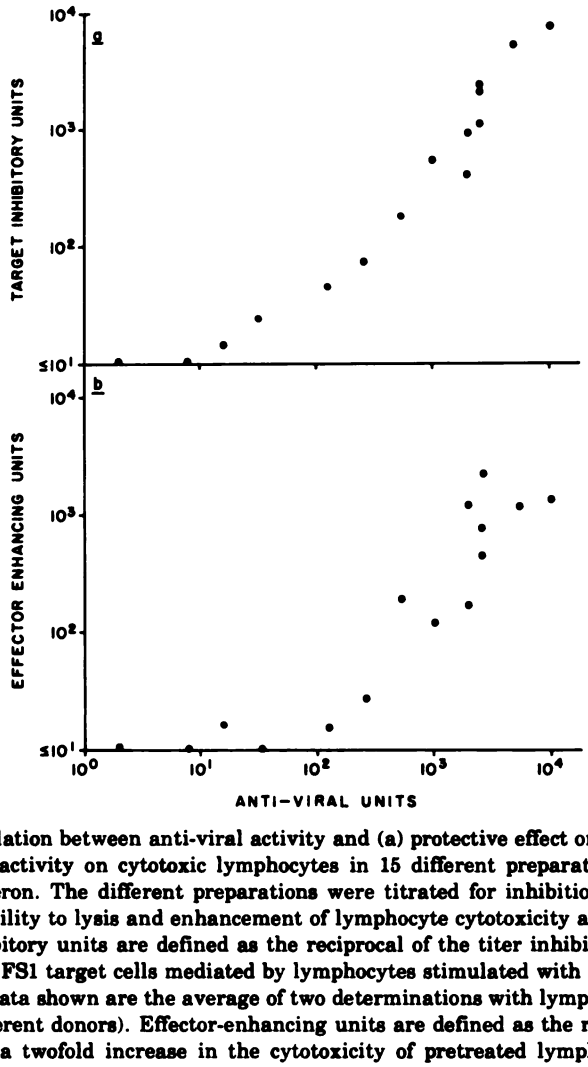

FIG. 5. Correlation between anti-viral activity and (a) protective effect on target cells or (b) enhancing activity on cytotoxic lymphocytes in 15 different preparations of RDMC-induced interferon. The different preparations were titrated for inhibition of target cell (FS1) susceptibility to lysis and enhancement of lymphocyte cytotoxicity as shown in Fig. 4. Target inhibitory units are defined as the reciprocal of the titer inhibiting 50% of the cytotoxicity on FS1 target cells mediated by lymphocytes stimulated with 10^3 anti-viral U of interferon (data shown are the average of two determinations with lymphocyte preparations from different donors). Effector-enhancing units are defined as the reciprocal of the titer inducing a twofold increase in the cytotoxicity of pretreated lymphocytes on FS1 target cells. Correlation between anti-viral activity and target inhibitory activity: $r = 0.945$, $P < 0.001$; correlation between anti-viral activity and effector enhancing activity: $r = 0.628$, $P < 0.01$.

The interferon-mediated inhibition of target cell susceptibility to lysis was dose dependent: 1-2 anti-viral U of interferon were required for 50% inhibition of cytotoxicity on FS1 target cells (Fig. 4 and 5a). Inhibition of cytotoxicity was observed with both NDV- and RDMC-induced lymphocyte interferon. The anti-viral activity and the target protective activity were tested in 15 RDMC-induced interferon preparations obtained with lymphocytes from different donors: a significant positive correlation was observed between the two activities ($r = 0.945$, $P < 0.001$; Fig. 5a). None of the control preparations described for effector-enhancing activity contained anti-viral activity, and neither they nor mouse interferon preparations were able to induce resistance in human target cells.

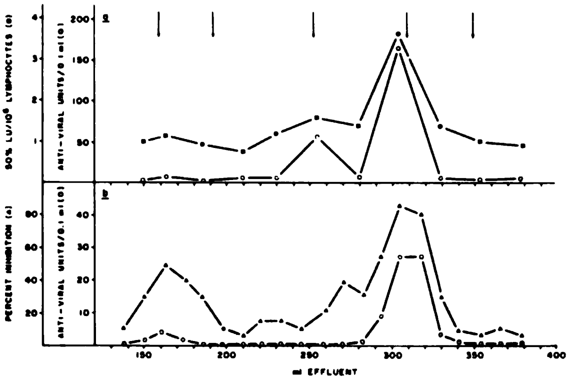

FIG. 6. Gel filtration on Sephadex G100 fine column of (a) RDMC-induced and (b) NDV-induced interferon preparations. 30 ml of preparation was concentrated ten times to 3 ml by vacuum dialysis. Column bed 25 × 960 mm (471 cm^3), void vol 160 ml. Arrows indicate molecular weight markers, from left to right: blue dextran 2,000 (mol wt 2,000,000), aldolase (158,000), ovalbumin (45,000), chymotrypsinogen A (25,000), ribonuclease A (13,700). The collected fractions were dialyzed against medium, sterilized by filtration, and tested for anti-viral activity, enhancing activity on cytotoxic lymphocytes (RDMC target cells, 8-h test, cytotoxicity of untreated lymphocytes: 1.04 lytic U/10^6 cells) and inhibitory activity on FS1 target cells (18-h pretreatment, 18-h test, effector lymphocytes stimulated with 10^3 anti-viral units of interferon).

TABLE II

Effect of Interferon on Lymphocyte Subpopulations

Fraction	ANAE*	S-Ig‡	E-RFC§	Ab-CMC‖	Sp-CMC¶	If-CMC**	If-CMC / Sp-CMC
Exp. A							
Ficoll-Hypaque separated cells	17.9	22.7	62.9	250	555	1,224	2.2
Nylon column nonadherent	1.4	2.4	86.4	909	1,111	3,571	3.2
Nylon column adherent	30.4	55.8	24.2	96	83	250	3.0
Exp. B							
Nylon column non-adherent	0.9	1.7	88.4	192	313	714	2.3
AET-SRBC Non-rosetting	11.4	32.0	0	526	1,562	4,348	2.8
AET-SRBC Rosetting	0	0	97.6	50	59	153	2.6

* Staining for nonspecific acid esterase (20), strongly positive cells (monocytes).

‡ Positive fluorescence for surface immunoglobulin with fluorescein-tagged rabbit $F(ab')_2$ anti-human light chains.

§ Cells forming rosettes with AET-treated sheep erythrocytes (18 h incubation at 4°C) (22).

‖ Lytic U/10^6 cells in antibody-dependent cell-mediated cytotoxicity.

¶ Lytic U/10^6 cells, 8 h test, RDMC target cells, effector cells preincubated 18 h in RPMI-FBS.

** Lytic U/10^6 cells, 8 h test, RDMC target cells, effector cells preincubated 18 h with 500 anti-viral U of RDMC-induced interferon.

The protective activity was resistant to pH 2.0 and destroyed by treatment with trypsin. Gel filtration on a Sephadex G100 column of NDV-induced interferon gave a peak of anti-viral and target protective activity with an approximate mol wt of 25,000 daltons; in addition, as discussed above, a variable proportion of the activity was eluted with the major protein peak (Fig. 6b).

Characteristics of Interferon-Mediated Target Cell Resistance to Cytotoxic Lymphocytes. FS1 target cells treated with 10^3 anti-viral U of interferon for 18 h were almost completely resistant to the cytotoxic effect of spontaneous

TABLE III

Effect of Interferon on Antibody-Dependent Cell-Mediated Cytotoxicity

Time in culture	Interferon	EB-P8 Target*	Increase‡	EB-P8 Target + Anti-HLA*	Increase‡
h					
0	0	30		2,564	
24	0	28	3.3	1,050	1.18
24	500	93		1,250	
48	0	24	12.6	952	1.31
48	500	303		1,250	

Lymphocytes were cultured in presence or absence of 500 anti-viral units of RDMC-induced interferon and tested at the different times in a 4-h assay against EB-P8 cells sensitized or not sensitized with a mixture of appropriate anti-HLA sera.

* Lytic U/10^6 cells.

‡ Ratio between lytic units of lymphocytes incubated in the presence and absence of interferon.

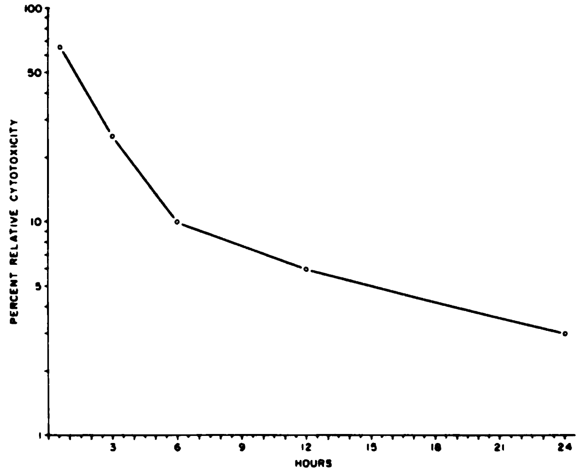

FIG. 7. Induction of target cell (FS1) resistance to lysis after pretreatment with interferon for different time periods. FS1 target cells were pretreated with 10^3 anti-viral U of RDMC-induced interferon for the times indicated, washed three times, and tested against interferon-stimulated (10^3 anti-viral U, 18-h pretreatment) cytotoxic lymphocytes. The solid dot indicates the cytotoxic effect on FS1 cells incubated for 30 min in the presence of interferon, washed and incubated without interferon for 24 h before testing. The relative cytotoxicity is expressed as the ratio between lytic units obtained against interferon-pretreated and untreated target cells.

(natural) cytotoxic lymphocytes and interferon-stimulated lymphocytes, but were only partially protected against PHA-induced cytotoxic human lymphocytes (Fig. 3). Moreover, in repeated experiments treated and untreated FS1 cells were equally susceptible to mouse anti-human cytotoxic T cells.

Interferon-treated and untreated unlabeled FS1 cells (cold targets) were tested for their ability to compete for the killing of [^{51}Cr]labeled FS1 cells, using interferon-stimulated human lymphocytes as effector cells. 5×10^4 cold target cells treated or not treated with interferon were added to the wells of microtiter

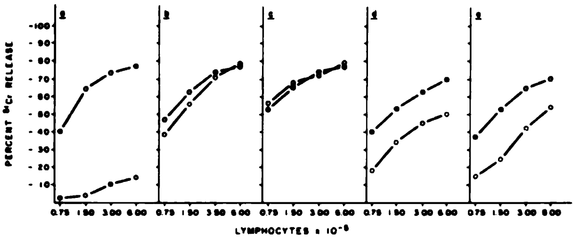

FIG. 8. Effects of metabolic inhibitors or virus infection on interferon-mediated inhibition of target cell (FS1) susceptibility to lysis. ●——●, FS1 cells not treated with interferon; ○——○, FS1 cells treated with interferon. a: FS1 cells were pretreated for 18 h with 10^3 anti-viral U of RDMC-induced interferon and then tested against different dilutions of interferon-stimulated (10^3 anti-viral units, 18 h pretreatment) cytotoxic lymphocytes. b,c: 1 h before and during interferon treatment 1 μg/ml of actinomycin D (b) or 50 μg/ml of cycloheximide (c) were added. d,e: 1 h before interferon treatment FS1 cells were infected with 0.1 ml (850 HAU/ml) of the HK strain of influenza A virus (d) or with 0.1 ml (10^6 TCID50/ml) of the Lister strain of vaccinia virus (e). The SD of each experimental point was less than 2%.

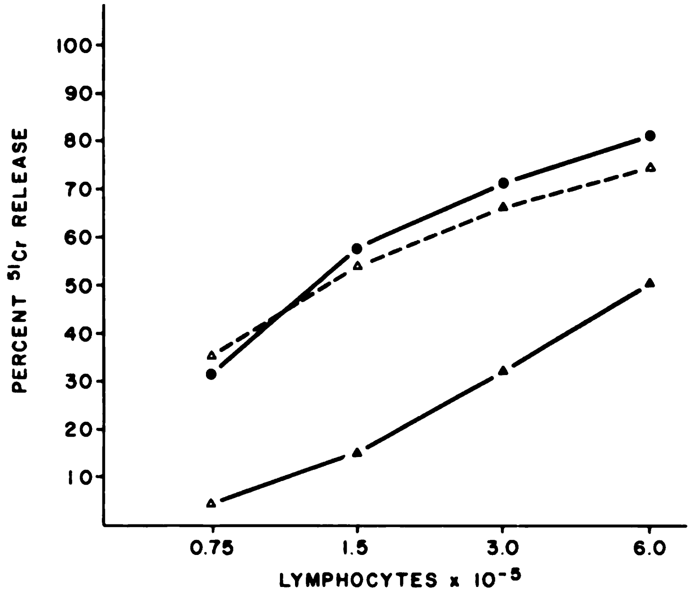

FIG. 9. Cold target competition experiment with interferon-treated or untreated competitor cells. 5 × 10^4 treated (10^3 anti-viral U, 18 h) or untreated FS1 cells were added to 2 × 10^4 adherent [^{51}Cr]labeled FS1 target cells. Effector cells were interferon-stimulated human lymphocytes; the incubation time of the cytotoxic test was 11 h. The SD of each experimental point was less than 2%. ●——●, no cold target cells; △-----△, interferon-treated FS1 cold target cells; ▲——▲, untreated FS1 cold target cells.

plates containing a monolayer of about 2 × 10^4 target cells labeled with ^{51}Cr. Different dilutions of effector lymphocytes were then added: as depicted in Fig. 9 about 75% inhibition of cytotoxicity was observed with untreated cold target cells, whereas no inhibition was obtained with interferon-treated cells.

Discussion

Since its disovery in 1957 by Isaacs and Lindenmann (29) as "an anti-viral substance produced by cells in response to virus infection", interferon has been shown to affect various cellular functions not directly connected with viral replication and to be induced by a variety of non-viral stimuli. In vivo interferon may regulate several cellular functions and defense mechanisms, and its anti-viral activity may represent only one aspect of its physiological role. Interferon is induced in immunological reactions, and it may either stimulate or depress several immune functions (1-9).

Interferon probably enhances a specific cell-mediated cytotoxic response against tumor antigens, in analogy to what has been demonstrated for alloreactive response (5, 6), and also stimulates nonspecific defense mechanisms such as phagocytosis (7, 8) and nonspecific cell-mediated cytotoxicity (9). Stimulation of the host defense mechanisms to more efficiently oppose tumor growth, and a direct effect on tumor cell DNA synthesis and replication, which decreases tumor growth rate and invasiveness, are probably responsible for the anti-tumor effect in vivo of interferon and interferon inducers (30). We have demonstrated that most tumor-derived cell cultures have the ability to induce human and mouse lymphocytes to produce interferon in vitro (20). This observation suggests that in vivo transplantable tumors used in experimental tumor immunology, and possibly spontaneous tumors, induce interferon, which, in the first phases of tumor growth, might stimulate host defense mechanisms.

The present study demonstrates that induction of lymphocyte interferon by tumor-derived cells affects the natural cytotoxic activity of human lymphocytes present in the cultures. Interferon preparations increase several fold the nonspecific cytotoxicity of human lymphocytes: the effector cells are cytotoxic for any target cell line tested, regardless of the cell line (or virus) used to induce interferon. Cell separation experiments showed that the activity of spontaneous (natural) killer lymphocytes could not be separated from the cytotoxic activity induced by interferon preparations. This observation suggests that interferon preparations do not generate a new population of effector cells, but rather enhance the activity of the natural killer cells or increase their number by recruiting quiescent cells. All the evidence indicates that in the interferon preparations it is the interferon itself that mediates the cytotoxicity-enhancing activity (31, 32). A very good correlation was found in both tumor cell-induced and virus-induced interferon preparations between anti-viral and cytotoxicity-enhancing activities. All control preparations were negative for both activities; moreover, the cytotoxicity-enhancing activity showed the species preference and the physicochemical characteristics of interferon.

The mechanism by which interferon enhances lymphocyte cytotoxicity is still obscure. Interferon does not act directly on target cells: interferon preparations are not toxic for target cells and the enhancement of cytotoxicity is present when lymphocytes are pretreated with interferon and washed before being tested as effector cells. The effect of interferon on cytotoxic lymphocytes is not immediate, but reaches a maximum after several hours; addition of interferon to a lymphocyte target cell mixture is less efficient than pretreating lymphocytes with interferon. Interferon therefore does not act by providing a recognition-binding system, as, for example, IgG molecules do in inducing antibody-dependent cell-mediated cytotoxicity, but directly stimulates effector cells. It is not possible to determine whether this stimulation requires active protein synthesis in cells, because although protein synthesis in the lymphocytes is not required for immediate cytotoxicity, pretreatment of lymphocytes with protein synthesis inhibitors determines a decrease of the cytotoxic efficiency (33 and our unpublished observation). This observation however suggests that cytotoxic activity of lymphocytes requires protein factors with a rapid turnover; interferon, which has been shown, for example, to increase the expression of H-2 antigens on murine cells (34), might affect the synthesis of such factors.

It has been suggested that human natural killer cell activity is partly mediated by IgG antibodies adsorbed to the Fc-receptor of antibody-dependent cytotoxic lymphocytes (19); in this case two mechanisms that might increase cytotoxicity would be an enhancement of the efficiency of antibody-dependent killer cells or an induction of in vitro synthesis of IgG able to arm killer cells. Both of these mechanisms seem to be excluded by the following observations (*a*) the activity of antibody-dependent killer cells is not enhanced by interferon (Table III); (*b*) interferon inhibits antibody secretion in several in vitro systems (4); (*c*) the cytotoxicity and the generation of interferon-induced killer cells is not affected by concentrations of rabbit $f(ab')_2$ fragment anti-human IgG able to completely suppress antibody-dependent cell-mediated cytotoxicity (25 and our unpublished observation).

The interferon-mediated enhancement of cytotoxicity in vitro lasted for 2–3 days. After 6–8 days of incubation the cytotoxicity of the untreated lymphocytes, which reached its lowest level at 4–5 days, increased again to levels close to or higher than those observed with freshly separated lymphocytes. This nonspecific increase of cytotoxicity, which parallels a simultaneous increase in [^{3}H]thymidine incorporation in the lymphocytes, is depressed by interferon. This phenomenon might be due to a polyclonal T-cell stimulation induced by mitogenic factors present in the culture medium or serum, however the nature of the effector cells involved has not been analyzed.

Various phenomena have been described which may be related to the cytotoxicity-enhancing activity of interferon. Peter et al. (35) reported that in mixed cultures of human lymphocytes and tumor-derived cell lines, a factor is released that inhibits DNA synthesis and enhances the spontaneous cell-mediated cytotoxicity of lymphocytes. That factor could be identical to the cell-induced lymphocyte interferon that we have described. Although Peter et al. considered the factor a lymphotoxin, no evidence of a direct toxic effect was presented. Several studies have shown that human lymphocytes kill virus-infected cells more efficiently than uninfected target cells (36–41): although anti-viral antibodies adsorbed on the membranes of the lymphocytes or secreted in the culture play a role in some systems (40, 41), lymphocytes cultured with virus-infected cells, or directly with virus, produce high level of interferon, which is the main factor responsible for the observed increase in cytotoxicity.[2] It has been reported that cytotoxic effector cells are generated by culturing human lymphocytes with tumor-derived cell lines (42–44). Although no direct analysis has been reported in these studies, we assume that in some of these systems the effector cells are probably induced by interferon released in the culture system. Porzsolt et al. (44) have described the generation of cytotoxic cells in a mixed culture of human lymphocytes and cultured melanoma cells with kinetics resembling that obtained with interferon stimulation (Fig. 2). Rapid induction of human cytotoxic lymphocytes in vitro has also been described with interferon inducers, such as synthetic polyribonucleotides (45) and Bacillus Calmette-Guérin (BCG) (46), and variously interpreted. In vivo it has been reported that injection of viruses (47) or BCG (47, 48) into mice increases the natural killer cell activity with a peak at day 3. Interestingly, the same phenomenon has been described after injection of transplantable tumor cell lines (47); some of these same cell lines have been tested in our laboratory and found to be able to induce interferon in vitro when cultured with human or mouse lymphocytes (20).

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[2] D. Santoli, G. Trinchieri, and H. Koprowski. Cell-mediated cytotoxicity in humans against virus-infected target cells. II. Interferon induction and activation of natural killer cells. Manuscript submitted for publication.

When target cells are treated with interferon, they are less susceptible to the cytotoxicity of effector lymphocytes. Interferon-treated fibroblasts are almost completely resistant to nonspecific cytotoxic lymphocytes. The refractory status of the target cells appears specific for the cytotoxicity mediated by natural killer cells; the spontaneous or interferon-enhanced cytotoxicity of human lymphocytes is almost completely suppressed, whereas the activity of mouse cytotoxic T cells is not affected. Human cytotoxic lymphocytes induced with PHA, which probably stimulated both cytotoxic T cells and natural killer cells (49), are only partially inhibited. Interferon therefore does not induce a general resistance in target cells: the inability of interferon-treated cold target cells to competitively inhibit the cytotoxicity of natural killer cells suggests that in the treated cells some surface characteristics responsible for recognition by cytotoxic lymphocytes are lost or masked. Although the phenomenon of the specific inhibition of the target cells cannot yet be interpreted, we feel that this observation may provide a clue for the specificity of the natural killer cells. Two considerations suggest that interferon does not act by affecting the expression of antigens coded by endogenous viruses present in the target cells and recognized by the cytotoxic cells (50): no evidence of the presence of virus or viral antigens in normal human fibroblasts have ever been obtained and interferon increases rather than decreases the expression of C-type virus antigens or particles on the surface of murine cells (51).

The identification of the molecules mediating the protection of target cells as interferon is based on the same characteristics already mentioned for the cytotoxicity-enhancing effect. Further, these traits meet the criteria established by Lockart (31) and Oxman (32) for the identification of interferon. The protection of target cells by interferon is not direct. Rather, the experiments with metabolic inhibitors suggest that interferon acts through an intracellular mechanism that involves synthesis by the cells of both RNA and protein. Infection with influenza A or vaccinia virus decreased or, in some experiments, abolished the induction of resistance to lysis in the target cells; this phenomenon, which has also been observed for the antiviral activity of interferon, may be due to the inhibition of host cell protein synthesis after infection with the two viruses tested (52, 53). The ability of different cell lines to respond to the anti-viral and to the target inhibitory activities of interferon were strictly correlated. Because cell susceptibility to other effects of interferon (e.g., inhibition of DNA synthesis and of cell growth) is not correlated with susceptibility to anti-viral activity, this correlation might indicate that the anti-viral and the target inhibitory effects of interferon follow a common intracellular pathway. Experiments with human-mouse somatic cell hybrids are in progress to test if products of the human chromosome 21, which presumably codes for the anti-viral protein (54), are responsible for the target inhibitory activity of interferon.

The protective effect of interferon on target cells offers a possible explanation of the paradoxical existence in vivo of natural killer cells, which, in vitro, can efficiently lyse any target cells, including normal autologous cells (13). Normal fibroblasts are more susceptible, in general, to the anti-viral and target cell inhibitory activities of interferon than cultured tumor cells or virus-infected cells. Interferon, by stimulating very efficient nonspecific cytotoxic cells and by simultaneously protecting normal cells from lysis, might render the natural killer cell system an inducible selective defense mechanism against tumor cells or virus-infected cells. Some tumor cell lines however (for example SW690 in Table I) maintained their susceptibility to the interferon target inhibitory activity: in this case interferon, by protecting the cells from lysis, may furnish an efficient escape mechanism to the tumor. A phenomenon of this type may perhaps explain why, in some transplantable tumor systems in mice, tumor cells cultured in vitro are more susceptible to killer cells than those grown in vivo (55, 56).

It is, however, difficult to speculate about the actual role that interferon has in vivo in regulating nonspecific defense mechanisms either in physiological conditions or during growth of spontaneous tumor and during viral infection. Mouse spleen cells from apparently healthy animals and, in some cases, human peripheral blood lymphocytes, spontaneously secrete low levels of interferon, suggesting that the presence of subliminal amounts of interferon are present in vivo even in physiological conditions. It is possible that the activity of the natural killer cells present in vivo is maintained by a continuous stimulation mediated by this low level of interferon. A better understanding of the role of interferon in vivo in modulating natural killer cell activity in physiological, pathological or experimental conditions must await further investigation.

Summary

Interferon, induced in lymphocytes either with viruses or cell lines, increases severalfold the natural cytotoxicity of human lymphocytes on target cell lines. Cell separation experiments support the hypothesis that interferon enhances the activity of natural killer cells rather than generating a new population of effector cells. In mixed culture of lymphocytes and cell lines in which endogenous interferon is produced, interferon mediates an enhancement of cytotoxicity that represents up to 70–90% of the observed cytotoxicity. The effect of interferon on target cells is antagonistic to the effect on the lymphocytes: the susceptibility

to cell-mediated lysis of various cells upon pretreatment with interferon is decreased and in some cases almost completely suppressed. Interferon renders target cells resistant to natural killer cells acting by an intracellular mechanism which requires RNA and protein synthesis. While normal fibroblasts are protected, virus-infected cells and most tumor cells usually are not protected by interferon. Interferon by stimulating very efficient nonspecific cytotoxic cells and by protecting at the same time normal cells from lysis, might render the natural killer cell system an inducible selective defense mechanism against tumor and virus-infected cells.

We would like to thank Doctors W. Gerhard, M. Herlyn, B. B. Knowles, Z. Steplewski, and T. J. Wiktor for kindly providing us with viruses and cell lines and Ms. Nancy Seitz, Patricia Radich, and Elsa Aglow for skillful technical assistance.

Received for publication 13 October 1977.

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SENSITIZATION OF LYMPHOCYTES AGAINST POOLED ALLOGENEIC CELLS

I. Generation of Cytotoxicity Against Autologous Human Lymphoblastoid Cell Lines*

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"Abnormal" cells such as vaccinia virus infected cells (1) and many tumor cells (2, 3), appear to express additional (as compared with normal cells of that strain or individual) antigens that are cross-reactive with alloantigens detected serologically on normal cells of other strains or individuals. Tumor cells may also express antigens cross-reacive with target antigens of foreign haplotypes against which cell-mediated immune responses can be generated (2, 4–6).

In vitro sensitization of responding human lymphocytes with a pool of allogeneic normal cells from 20 individuals results in the generation of cytotoxic T lymphocytes that lyse target cells from individuals that differ from the responding individual with regards to determinants recognized by cytotoxic lymphocytes, i.e. CD antigens[1] (7). Included in such an allogeneic pool of lymphocytes may be essentially all the CD-like antigens of the species. Whether sensitization with the pool may be an effective means to generate cytotoxic cells directed against syngeneic or autologous abnormal target cells could be tested by using human lymphoblastoid cell lines (LCL) derived from lymphocytes transformed by Epstein-Barr virus (EBV) since LCL cells express target antigens recognized by lymphocytes sensitized against autologous LCL cells (8, 9).

The results reported herein demonstrate that sensitization of lymphocytes with the pool of allogeneic normal cells gives rise to effector cells cytotoxic to autologous LCL cells but not to autologous normal lymphocytes nor phytohemagglutinin (PHA) induced blasts. In contrast, sensitization of lymphocytes with cells from single allogeneic individuals rarely leads to the generation of effector cells cytotoxic for autologous LCL cells.

Materials and Methods

Lymphocytes were isolated from heparinized peripheral blood from normal healthy adults by Ficoll-Hypaque sedimentation. For some experiments, lymphocytes were fractionated into T-cell-enriched and B-cell-enriched populations by rosetting with sheep erythrocytes (SRBC), centrifuging on a Ficoll-Hypaque gradient, and isolating the cells at the interface (depleted of T cells) and

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* Supported in part by National Institutes of Health grants CA-20409, CA-14520, CA-16836, AI-11576, AI-08439 and National Foundation-March of Dimes grants CRBS 246 and 6-76-213; paper no. 2191 from the Laboratory of Genetics and paper no. 142 from the Immunobiology Research Center, The University of Wisconsin, Madison, Wis. 53706.

[1] *Abbreviations used in this paper*: LCL, lymphoblastoid cell lines; EBV, Epstein-Barr virus; CD antigens, those antigens detected by cytotoxic lymphocytes.

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TABLE I

*Lysis of Autologous and Allogeneic LCL Cells after Pool Sensitization**

Responder cells	Stimulating cells	Number of effector cells per target cell	Target cells					
			A-LCL	A	S-LCL	S	C-LCL	C
			% Specific ^{51}Cr release (±SD)					
A	Pool$^{20}_{x}$	35	46.7 ± 5.7	−2.8 ± 3.7	92.3 ± 7.6		87.4 ± 6.5	
A	A-LCL$_x$	60	41.1 ± 19.1					
S	Pool$^{20}_{x}$	35	82.4 ± 9.2		17.9 ± 3.3	2.8 ± 3.3	85.9 ± 7.2	64.5 ± 3.4
S	S-LCL$_x$	60			33.4 ± 2.4			
C	Pool$^{20}_{x}$	35	94.0 ± 11.8	70.1 ± 9.1	91.4 ± 7.6	44.3 ± 5.4	5.3 ± 3.2	−2.8 ± 1.6
C	C-LCL$_x$	60					70.2 ± 3.5	

* Lymphocytes from individuals A, S, and C were stimulated with X-irradiated autologous LCL cells (designated A-LCL, S-LCL, or C-LCL) or a pool of 20 allogeneic stimulating cells and lysis of the LCL cells and normal lymphocytes (A, S, and C) was measured on day 7. Lymphocytes from A, S, and C cultured in media alone caused −3.0–2.3% ^{51}Cr release from autologous LCL cells.

the pellet containing rosetted cells (more than 95% T cells). The micromethod used for generating and assaying allogeneically induced cytotoxic human lymphocyte responses in vitro has been recently described (10). In brief, 1 × 10^5 responding lymphocytes/well were cultured with 1 × 10^5 X-irradiated (2,500 rads) stimulating cells from single unrelated individuals, with "pool" stimulating cells prepared by pooling equal numbers of lymphocytes from 5–20 unrelated individuals (7, 11), or with 2 × 10^4 X-irradiated (4000 rads) autologous LCL cells. LCL cells were generated by infecting peripheral blood lymphocytes with EBV (12) and were grown in RPMI-1640 containing 25 mM Hepes buffer and 10% heat inactivated normal human serum; the LCL cells were used for experiments within 6 mo after their establishment and were found to be free of mycoplasma contamination. In some experiments, effector cells were generated in upright 50 ml tissue culture flasks as previously described (13) by using 8 × 10^6 responding lymphocytes and 8 × 10^6 X-irradiated pooled allogeneic stimulating cells or 1.5 × 10^6 X-irradiated autologous LCL cells. ^{51}Cr release assays were performed 7 days after the onset of mixed culture and were terminated after 7 h. The percent specific ^{51}Cr release was calculated as previously described (10). All sensitizations and cytotoxic assays were performed in medium consisting of RPMI-1640 containing 25 mM Hepes Buffer and 20% heat-inactivated normal human serum.

Results

Shown in Table I are results of an experiment in which lymphocytes of three different individuals were cultured with X-irradiated cells of a pool consisting of 20 members for 7 days and were tested for their ability to lyse allogeneic LCL cells, autologous LCL cells, and autologous cultured normal lymphocytes. Lymphocytes from individuals A and S after pool sensitization significantly lysed autologous LCL cells as well as allogeneic LCL cells. Pool sensitized lymphocytes, however, failed to kill autologous normal lymphocytes, consistent with the findings of Martinis and Bach (7). Although LCL cells from individual C stimulated autologous lymphocytes to differentiate into cytotoxic lymphocytes capable of lysing the autologous LCL cells (as did autologous sensitization with A and S), pool sensitization of lymphocytes from individual C did not lead to significant lysis of autologous LCL cells. Lymphocytes from individuals A, S, and C when cultured without stimulating cells failed to kill autologous LCL cells.

Whereas pool sensitization is an effective means for generating T lymphocytes cytotoxic for autologous LCL cells in three of the four individuals tested, cultured autologous unfractionated lymphocytes, lymphocytes enriched for T

TABLE II

*Ability of Pool Sensitized T Lymphocytes to Lyse Autologous LCL Cells and Their Failure to Lyse Autologous B Cells, T Cells, and PHA Blasts**

	Responding T lymphocytes	Stimulating cells	Target cells				
			H-LCL	H-T Cells	H-B Cells	Allogeneic LCL	
			% specific ^{51}Cr release (±SD)				
Exp. 1	H	Pool$^{20}_{x}$	20.8 ± 8.9	−1.8 ± 9.4	2.9 ± 9.7	35.9 ± 5.2	
	D	H_x		61.9 ± 6.9	65.2 ± 11.6		
Exp. 2			S-LCL	S-Cultured lymphocytes	S-PHA Blasts	M-Cultured lymphocytes	M-PHA Blasts
	S	Pool$^{20}_{x}$	15.3 ± 3.1	−2.7 ± 2.0	−1.3 ± 3.9	23.9 ± 5.0	13.3 ± 4.7
	M	Pool$^{20}_{x}$		43.0 ± 5.6	47.2 ± 4.8	−0.5 ± 3.7	−4.3 ± 1.7

* Responding T lymphocytes were stimulated with X-irradiated pool of 20 allogeneic cells or X-irradiated cells of individual H. CML assays were performed on day 7 by using 40 effector cells:target cells. T-enriched and B-enriched cells from individual H (designated H-T and H-B) were isolated and cultured for 7 days before use as target cells. PHA blasts were used 60 h after incubating lymphocytes with PHA.

cells or B cells, and autologous PHA blasts are not lysed by the pool sensitized cells (Table II). Results of Exp. 1 show that sensitization of T cells from individual H gave rise to CTLs capable of lysing allogeneic as well as autologous LCL cells but not autologous normal T-enriched nor normal B-enriched cells, that were, however, sensitive to lysis by T cells from individual D after stimulation with cells of individual H. Similar results were obtained in another experiment. Shown in Exp. 2 is the finding, with appropriate controls, that T cells of individuals after pool sensitization lysed autologous LCL cells but failed to lyse autologous normal lymphocytes or PHA blasts.

Shown in Table III are results of two experiments to determine whether sensitization of lymphocytes with cells from individual members of the pool would be equally efficacious in terms of generating cytotoxic lymphocytes directed against autologous LCL cells. In Exp. 1, lymphocytes from individual S were sensitized in separate mixed cultures with cells of 10 unrelated individuals, designated 1–10, with three different pools of allogeneic normal cells consisting of 5, 10, and 20 members, respectively, and with autologous LCL cells. No cytotoxicity for LCL cells was observed on day 5 or 6 (data not shown). Stimulation of S with cells of individual 2 resulted, by day 7, in the generation of cytotoxic cells that lysed autologous LCL cells, however, very low or insignificant amounts of lysis of the LCL cells occurred by lymphocytes sensitized to any of the other nine individuals. In contrast, sensitization of S with the pool of cells from 20 members resulted in the same level of cytotoxicity on autologous LCL cells as did sensitization with S's own LCL cells and resulted in a greater cytotoxic response than did sensitization with pools consisting of cells from 5 or 10 members.

In a second experiment, shown in Table III, sensitization of cells of individual S to a pool of either 8 or 18 members resulted in significant cytotoxicity for his autologous LCL cells as well as target cells from all 6 members of the pool tested. In contrast, effector cells generated in each of the eight individual mixed cultures did not lyse autologous LCL cells in any of the eight cases although

TABLE III

Lysis of Autologous LCL Cells and Allogeneic Cells after Allo- or Pool-Sensitization

Exp. 1			Exp. 2 Target cells						
Effector cells*	% specific ^{51}Cr release on S's LCL target cells‡	Effector cells	S's LCL cells	A	B	D	E	G	H
			% specific ^{51}Cr release ± SD‖						
S(1)$_x$	+4.7	S(A)$_x$	0.0 ± 2.7	33.5					
S(2)$_x$	+17.5	S(B)$_x$	1.9 ± 3.0		43.7				
S(3)$_x$	+3.9	S(C)$_x$	2.8 ± 3.9						
S(4)$_x$	−2.7	S(D)$_x$	1.9 ± 4.3			50.9			
S(5)$_x$	+9.1	S(E)$_x$	3.7 ± 4.4				92.9		
S(6)$_x$	+5.8	S(F)$_x$	0.1 ± 2.7						
S(7)$_x$	+5.3	S(G)$_x$	−0.8 ± 3.8					39.3	
S(8)$_x$	−0.7	S(H)$_x$	2.3 ± 5.0						37.1
S(9)$_x$	0	S(Pool^8)$_x$	18.7 ± 3.2			53.9			
S(10)$_x$	0	S(Pool^{18})$_x$	23.0 ± 2.6	18.5	30.3		101.6	45.9	112.9
S(pool^5)$_x$	+9.6	S(S-LCL)$_x$	24.6 ± 3.1						
S(pool^{10})$_x$	+16								
S(pool^{20})$_x$	+22.3								
S(S-LCL)$_x$	+20.5								

* Lymphocytes were sensitized with X-irradiated normal lymphocytes from individuals 1–10, with pooled allogeneic cells from 5, 10, or 20 members (designated pool^5, pool^{10}, pool^{20}) or autologous LCL cells in microwells for 7 days.

‡ Standard deviations of the percent ^{51}Cr release ranged from 1.4 to 5.2%.

§ Lymphocytes were sensitized with X-irradiated normal lymphocytes from individuals designated A–H, with pooled cells from 8 of 18 individuals, or with autologous LCL cells and cytotoxicity on the allogeneic target cells derived from members of the pool and autologous LCL cells were assayed on day 7 by using a ratio of 25 effector cells:target cell.

‖ SD ranged from 1.1 to 6.2% ^{51}Cr release.

these same effector cells were highly cytotoxic for target cells derived from the sensitizing cell donors.

Since sensitization of lymphocytes from S with cells from individual 2 (Table III) resulted in the generation of cytotoxic cells directed against autologous LCL cells, cold target inhibition studies were performed to determine whether lysis of LCL cells after pool sensitization was due solely to the presence of cells from individual 2 in the pool. When cells of S were sensitized to the pool and then tested for their ability to lyse ^{51}Cr-labeled autologous LCL cells, the addition of unlabeled LCL cells blocked lysis whereas unlabeled target cells from individual 2 did not (Fig. 1A). In contrast, when lymphocytes from S were sensitized with cells from individual 2, lysis of the autologous LCL cells was blocked equally well with unlabeled target cells from individual 2 or unlabeled LCL cells (Fig. 1B). Lysis of target cells from individual 2 by lymphocytes sensitized to the pool or to cells of 2 was not blocked by the LCL cells but was by unlabeled target cells from individual 2.

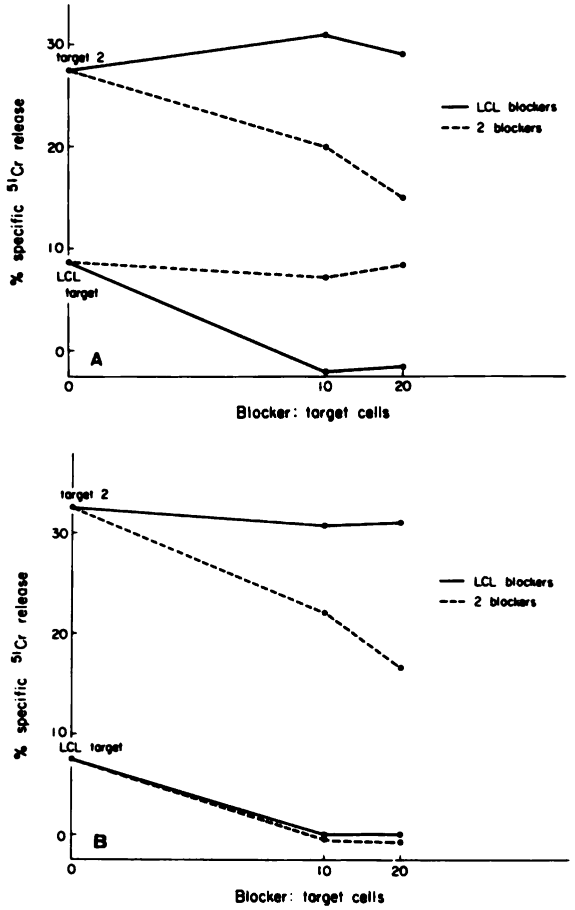

FIG. 1. Ability of unlabeled cells to block ^{51}Cr release from LCL cells of S on target cells from individual 2. Lymphocytes from individual S were sensitized to a pool of 20 members (A) or to cells from individual 2 (B) and were tested for their ability to lyse ^{51}Cr labeled LCL cells or target cells from individual 2 at a ratio of 30 effector cells: target cell in the presence of varying numbers of unlabeled ("blockers") LCL cells or cells from individual 2.

Discussion

The data presented in this paper demonstrate that in vitro sensitization of human lymphocytes with a pool of allogeneic normal human lymphocytes results in the development of effector cells that lyse autologous EBV transformed lymphoblastoid cells in three of four individuals tested. We have extended our previous findings (7) by demonstrating that T cells can respond to allogeneic pooled cells by differentiating into CTLs capable of lysing allogeneic normal lymphocytes, LCL cells, and PHA blasts but not autologous cultured lymphocytes enriched for T cells or B cells, nor autologous PHA blasts.

An occasional allogeneic normal cell appears to carry determinants cross-reactive with target antigens on autologous LCL cells although most allogeneic normal cells would not appear to do so. Sensitization with the cells of individual 2 (Table III and Fig. 1B) resulted in lysis of LCL cells autologous with the responding cell donor. Results of blocking experiments demonstrated that lymphocytes sensitized to cells of individual 2 were not blocked from lysing 2's target cells by LCL cells (see Fig. 1B) and that after pool sensitization, lysis of the LCL cells was blocked only by LCL cells and not by cells of individual 2 (see Fig. 1A). We would interpret these findings to indicate that target antigens on the autologous LCL cells include determinants in addition to those shared by individual 2, and that the shared determinants represent a minority of all those that can be recognized by S's lymphocytes after pool sensitization.

It would seem that there are at least three mechanisms by which antigens expressed on LCL cells might cross-react with alloantigens. First, as a result of EBV infection or morphological transformation there may be derepression of genes coding for antigens that are expressed on normal cells of other members of the species (14, 15). Second, EBV may code for cell membrane determinants that partially cross-react with many alloantigens. Third, any genetic or phenotypic modification of CD antigens might result in expression of target antigens that would be cross-reactive with alloantigens (16). Use of the pool may have general applicability in terms of generating effector cells cytotoxic for autologous or syngeneic virus infected or morphologically transformed cells (16).

Summary

Lymphocytes sensitized in vitro to a pool of X-irradiated allogeneic normal lymphocytes from 20 individuals develop cytotoxic activity for autologous human lymphoblastoid cells (LCL). Whereas pool sensitized T lymphocytes lyse autologous LCL cells, they fail to lyse autologous B-enriched or T-enriched normal target cells nor autologous phytohemagglutinin (PHA) blasts. In contrast to pool sensitization, stimulation with normal cells of single allogeneic individuals rarely led to development of cytotoxicity against autologous LCL cells. We conclude that human Epstein-Barr virus transformed LCL cells express target antigens cross-reactive with allogeneic target antigens expressed on normal cells and that sensitization with a pool of allogeneic cells is an effective means of generating effector cells directed against autologous abnormal cells.

We thank Vance Baker for excellent technical assistance, Andrew Watson for helpful discussions, and Karen Heim for help in preparation of this manuscript.

Received for publication 3 January 1978.

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ISOLATED HAPTEN-BINDING RECEPTORS OF SENSITIZED LYMPHOCYTES

III. Evidence for Idiotypic Restriction of T-Cell Receptors*

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There is now good evidence that variable portions of both T- and B-cell receptors for antigen are encoded by *V* genes in the heavy chain linkage group (1, 2, 3). However, it is not known whether the sets of *V* genes expressed in the two cell compartments are identical or only overlapping. Only a few *V* genes, identified by serological markers of their gene product (idiotypic markers), have been shown to be coexpressed in both T- and B-cell receptors, and indirect evidence from functional and regulatory studies would suggest that in the two cell compartments, the rules by which cells are selected differ according to their receptor specificity (4).

In the present study we approach this question in a more direct way. The humoral antibody response of C57BL/6 mice against the hapten 4-hydroxy-3-nitro-phenylacetyl (NP)[1] is in its initial phase restricted to a family of closely related antibody species which constitute the NP^b idiotype. This idiotype is inherited as a single genetic unit in close linkage to the heavy chain allotype (5, 6). In the secondary immune response, the proportion of antibodies carrying the NP^b idiotype declines from 70–90% to 10–20%, and the antibody population becomes strongly heterogeneous (7, 8). Our technique of receptor isolation from T and B lymphocytes (3, 9–12) allows us to study idiotype expression at the receptor level in parallel. We show here that NP-binding receptors isolated from sensitized B cells are strikingly similar to humoral antibodies in the secondary response in that only a small fraction expresses the NP^b idiotype. In contrast, receptors of putative T-cell origin appear to be restricted to the primary NP^b idiotype in accord with our previous data (3).

Materials and Methods

Animals. C57BL/6 mice and BALB/c mice were obtained from the Zentralinstitut für Versuchstierzucht, Hannover, West Germany, and Gl. Bomholtgaard, Ltd., Ry, Denmark. CBA mice were purchased from The Jackson Laboratory, Bar Harbor, Maine, and from S. Ivanovas, Ltd., Kisslegg, West Germany. (C57BL/6 × CBA/J)F_2 hybrids were bred in our animal colony.

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* Supported by the Deutsche Forschungsgemeinschaft through Sonderforschungsbereich 74.

[1] *Abbreviations used in this paper*: cap, ε-aminocaproic acid; CG, chicken gamma globulin; G anti-MIg, goat anti-mouse Ig serum; HPI, haptenated phage inactivation; NIP, 4-hydroxy-5-iodo-3-nitro-phenylacetyl; NP, 4-hydroxy-3-nitro-phenylacetyl; R anti-Id, rabbit antiserum against NP^b idiotype.

Male and female mice, aged 6–8 wk, were vaccinated against ectromelia and rested for at least 2 wk before entry into the experiment.

Antisera and Immunosorbents. The polyspecific goat antiserum to mouse immunoglobulin (G anti-MIg) has been described (3, 9). An anti-idiotypic antiserum against primary anti-NP antibodies (the NP^b idiotype) of C57BL/6 mice was raised in a rabbit. For this purpose, C57BL/6 anti-NP antibodies were induced and purified by affinity chromatography as described previously (5). The rabbit was given an initial intramuscular injection of 100 μg of the antibodies in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) on the shoulder, followed by the injection of 50 μg antibody in incomplete adjuvant 1 mo later. After another 10 days, the rabbit received 50 μg of the antibody preparation i.v., and was bled 20 days after this injection. The resulting antiserum was absorbed on insolubilized normal C57BL/6 serum and MOPC 104e protein as described previously (5). We obtained an anti-idiotypic serum (R anti-Id) which reacted specifically with the majority (>80%) of primary C57BL/6 anti-NP antibodies, but did not bind C57BL/6 normal Ig or antibodies of unrelated specificity. In genetic experiments, the antiserum was shown to detect an idiotypic marker indistinguishable from the one detected by our previous guinea pig anti-idiotypic serum (5) except that the reaction of R anti-Id with the anti-NP antibodies could be specifically inhibited by 4-hydroxy-5-iodo-3-nitrophenylacetyl caproic acid (NIP-cap). The detailed characterization of R anti-Id is the subject of a separate publication.[2] The specificity of the antiserum, however, is apparent from the data reported in this paper (see Results). Immunosorbents were prepared by coupling the immunoglobulin (Ig) fraction of R anti-Id (prepared by precipitation with ammonium sulfate at 50% saturation) or G anti-MIg whole serum to Sepharose 4B-Cl (Pharmacia Fine Chemicals, Uppsala, Sweden) according to March et al. (13).

Immunizations, Receptor Preparations, Absorptions, and Phage Inactivation Assay (HPI). Preparation of NP_{16} chicken gammaglobulin (NP_{16}-CG), immunization procedures, preparation of hapten-coupled nylon discs, preparations of splenic lymphocytes, isolation of hapten-specific receptor material, absorption techniques, and HPI with NIP-cap-T4 bacteriophage (gift of Doctors O. Mäkelä and M. Becker) have been described previously (3, 9).

Results

The aim of the experiments described here was the determination of the frequency of the NP^b idiotype in NP-specific T- and B-cell receptors from mice carrying the $Ig\text{-}1^b$ allotype. For this purpose mice of various strains were primed with NP-CG, and NP-binding receptor material was isolated from the spleen cells on haptenated nylon mesh as described previously (3, 9).

The receptor material consists of two fractions, one reacting with class- and type-specific anti-Ig sera (anti-Ig^+ fraction), and another lacking determinants of constant Ig domains (anti-Ig^- fraction). Cell separation experiments indicate that the anti-Ig^+ fraction represents B-cell receptors, whereas the anti-Ig^- fraction originates from T lymphocytes (3, 9). The frequency of the NP^b idiotype in the two fractions was determined in a two-step procedure. First, the total receptor preparation was absorbed with insolubilized R anti-Id or insolubilized normal rabbit serum, and the hapten binding activity was determined before and after absorption. Assuming a similar avidity distribution in NP^b idiotype positive and negative molecules, the NP^b idiotype frequency in the total receptor material (anti-Ig^+ and anti-Ig^- fraction) can be calculated from these data. Second, the anti-Ig^- fraction was separated from the total receptor preparation by absorption with insolubilized G anti-MIg serum and the frequency of the NP^b idiotype was determined in this fraction as describod above. The combined data also allow the calculation of the idiotype frequency in the anti-Ig^+ fraction.

The results of a typical experiment appear in Fig. 1. In this experiment,

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[2] T. Imanishi-Kari and K. Rajewsky. Manuscript in preparation.

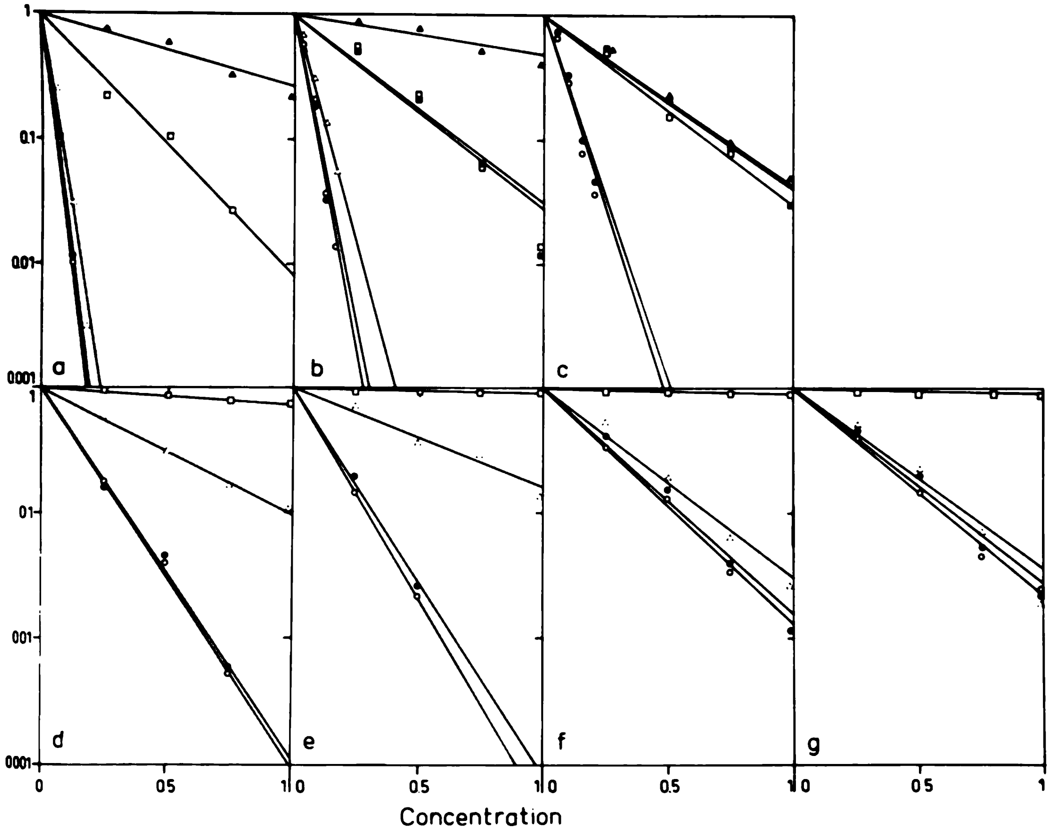

FIG. 1. Analysis of idiotype expression in receptor material and humoral antibodies. The following receptor preparations isolated on NP-cap nylon discs from spleen cells of NP_{16}-CG sensitized mice and anti-NP serum antibodies of the lymphocyte donor strains were analysed: (a) C57BL/6 receptor isolated 6 wk after primary immunization, (b) (C57BL/6 × CBA/J)F_2 receptor isolated 4 wk after primary immunization from animals selected for homozygous Ig-1^b allotype, (c) BALB/c receptor isolated 13 wk after primary immunization, (d) serum pool collected from 10 C57BL/6 mice bled 6 wk after priming, (e) primary C57BL/6 antibodies purified by adsorption to NP-bovine serum albumin-Sepharose and subsequent elution with acidic buffer, (f) serum pool collected from 5 C57BL/6 mice bled 10 days after secondary immunization, (g) serum pool collected from 7 BALB/c mice bled 6 wk after primary immunization. The concentration of phage inactivating material on the abcissa is given in arbitrary units. Inactivation of NIP-cap-T4 bacteriophage (p/p_0, number of bacteriophage plaques in presence of inactivator divided by number of plaques in absence of inactivator) by (○) nonabsorbed material or material absorbed on: (●) Sepharose-bound normal rabbit serum; (□) G anti-MIg immunosorbent; (△) R anti-Id immunosorbent; (■) G anti-MIg immunosorbent in two sequential absorptions steps; (▲) G anti-MIg immunosorbent and subsequently on R anti-Id immunosorbent. Immunosorbents were prepared by coupling whole normal serum, G anti-MIg serum or the IgG fraction of R anti-Id serum to CNBr-activated Sepharose 4B-CL.

receptors from three groups of animals were analyzed. Two receptor preparations originated from mice carrying the Ig-1^b allotype, namely C57BL/6 mice and mice from a (C57BL/6 × CBA)F_2 generation which were homozygous for the Ig-1^b allotype. We know from previous experiments which included a genetic analysis that the anti-Ig^- fraction in these two receptor preparations carries the

TABLE I

Binding of NP-Specific Receptors and Serum Antibodies to G anti-MIg and R anti-Id Immunosorbents

No.	NP-binding material	Total receptor and antibodies		Anti-Ig$^-$ fraction‡	Anti-Ig$^+$ fraction
		% Absorption by G anti MIg	% Absorption by R anti Id	% Absorption by R anti-Id*	
1	C57BL/6 receptor	85§	20	74	9
2	(C57BL/6 × CBA)F_2 receptor, Ig-1$^{b/b}$	84‖	26	81	13
3	BALB/c receptor	75‖	n.d.¶	4	
4	Primary C57BL/6 anti-NP serum	96§	67		
5	Purified primary C57BL/6 anti-NP antibodies	99§	75		
6	Secondary C57BL/6 anti-NP serum	99§	18		
7	Primary BALB/c anti-NP serum	98§	8		

* Measured directly for anti-Ig$^-$ fraction and calculated for anti-Ig$^+$ fraction as described in text.

‡ The anti-Ig$^-$ fractions in the present study are smaller than in previous experiments. The reason for this discrepancy is not entirely clear but appears to be related to the technique for immunosorbent preparation in that certain types of immunosorbents of anti-Ig sera exhibit substantial nonspecific absorption.

§ Determined by single absorption with G anti MIg and corrected for absorption by normal rabbit serum.

‖ Determined by two sequential adsorptions with G anti-MIg and corrected for absorption on normal rabbit serum.

¶ n.d., not determined.

NP^b idiotype (3). The third receptor preparation was of BALB/c origin and should therefore be negative for the NP^b idiotype (5). Also included in the experiment were primary and secondary serum antibodies from NP-sensitized C57BL/6 mice, and primary anti-NP antibodies of BALB/c origin for control.

From the phage inactivation curves in Fig. 1 we can calculate the fractions of NP-binding activity associated with the NP^b idiotype in the various receptors and antibodies. The results of this calculation appear in Table I.

As expected, the frequency of the NP^b idiotype is high in primary and low in secondary anti-NP sera of C57BL/6 mice. Only 8% of the activity of BALB/c antibodies are absorbed by the anti-idiotypic serum. This must be considered nonspecific since in binding inhibition experiments, the same antibodies are totally negative for the NP^b idiotype (idiotype frequency <1%; T. Imanishi-Kari, unpublished data).

In the receptor preparations, the situation is strikingly different for anti-Ig$^+$ and anti-Ig$^-$ fractions. In accord with our previous data (3), the bulk of the activity of the anti-Ig$^-$ fraction from Ig-1^b mice is eliminated by the anti-idiotypic immunosorbent. Elimination is specific since it does not extend to anti-Ig$^-$ receptors of BALB/c origin. In contrast, the anti-Ig$^+$ receptor fraction from Ig-1^b mice appears to contain very few, if any, NP-binding molecules carrying the NP^b idiotype. This result is new and allows us for the first time to distinguish the anti-Ig$^-$ receptor fraction positively from the anti-Ig$^+$ fraction by a marker which is strongly expressed in the former, but not expressed, or scarcely detectable in the latter fraction. In our previous experiments (3), an anti-idiotypic serum was used which allowed the determination of the NP^b idiotype only in anti-Ig$^-$ receptors.

Discussion

The present study confirms by absorption analysis that the NP^b idiotype is expressed on the majority of primary anti-NP antibodies of C57BL/6 mice, but that it represents only a minor component of the antibody population in the secondary response (7, 8). Receptors on memory B cells in NP-sensitized animals should therefore be largely devoid of the NP^b idiotype. This is experimentally approached in our analysis of the anti-Ig$^+$ receptor fraction. The discovery of very low idiotype frequency in this material strongly supports our interpretation that the anti-Ig$^+$ fraction represents receptors of sensitized B lymphocytes. Since the majority of the anti-NP antibodies circulating in the animals from which the lymphocytes for receptor preparation are recovered carry the NP^b idiotype, the anti-Ig$^+$ receptor fraction cannot be passively adsorbed antibedy.

In striking contrast to the anti-Ig$^+$ fraction, the majority of the anti-Ig$^-$ receptors carry the NP^b idiotype as shown in the present and a previous study (3). The anti-Ig$^-$ fraction is considered to represent T-cell receptors on the basis of cell fractionation experiments (3, 9). Previous evidence argues against the possibility that this material is passively adsorbod conventional antibody in that the material does not appear to carry antigenic determinants of any of the known immunoglobulin constant domains (3, 11). Another strong argument comes from recent experiments with SJL mice.[3] These animals carry the Ig-1^b allotype, but do not detectably express the NP^b idiotype at the antibody level, presumably because of their low expression of lambda light chains which are associated with antibody molecules carrying the NP^b idiotype (5, 8, 14). NP-binding receptors isolated from sensitized SJL mice do not detectably express the NP^b idiotype in the anti-Ig$^+$ fraction, but most of the activity in the anti-Ig$^-$ fraction is specifically absorbed by the anti-idiotypic serum.

It would thus appear that in the present system, V_H gene expression in T-cell receptors is largely restricted to the major idiotype of the primary anti-NP antibedy response. The extent of this restriction is not clear at present. The T-cell-receptor repertoire might become more heterogeneous still later in the immune response, and we do not know whether the receptor molecules carrying NP^b idiotypic determinants are equally heterogeneous as the corresponding antibody molecules in the primary response (15).

The present experiments raise the question whether as far as V_H-gene expression goes, T cells are restricted to the expression of only a few genes which appear at the antibedy level as major idiotypes, and which, there is good reason to believe, are encoded in the germ line. B cells may initially express the same genes in their receptors, but then go through a diversification process in ontogeny. This would be in line with experimental results suggesting that the affinity of T-cell receptors for antigen does not increase in the course of an immune response, in contrast to the affinity of B-cell receptors (16, 17). It is tempting to ask whether the preferential recognition by T cells of structures encoded by the major histocompatibility complex might correspond to properties of the V^H domains to the expression of which T cells appear to be restricted. Experiments along these lines are in progress.

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[3] U. Krawinkel, M. Cramer, I. Melchers, T. Imanishi-Kari, and K. Rajewsky. Manuscript in preparation.

Summary

The primary antibody response of C57BL/6 mice to the 4-hydroxy-3-nitrophenylacetyl (NP) hapten is restricted to antibody molecules expressing the NP^b idiotype. This idiotype is a genetic marker for *V* genes in the heavy chain linkage group. In the secondary response, the frequency of NP^b idiotype-positive molecules within the antibody population drops to very low values. Accordingly, isolated NP binding receptors from NP-sensitized B lymphocytes are largely devoid of this idiotype. In contrast, the NP^b idiotype is expressed on the majority of the receptor fractions which we consider T-cell derived. This finding suggests that the antigen receptors of T lymphocytes may be restricted to the expression of major (germ-line encoded?) heavy chain idiotypes.

We thank Ms. C. Schenkel, Mrs. G. Giels, and Ms. S. Irlenbusch for their expert technical help.

Received for publication 10 January 1978.

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LOCALIZATION OF T25 GLYCOPROTEIN IN WILD-TYPE AND THY 1⁻ MUTANT CELLS BY IMMUNOFLUORESCENCE AND IMMUNOELECTRON MICROSCOPY*

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The steps in the biosynthesis of cell membrane glycoproteins and their transport to the cell surface are not well understood. We have been studying a series of mutant cell lines which have blocks in their cell surface expression of T25, a membrane glycoprotein bearing the Thy 1 alloantigen (1, 2). Biochemical characterization of the nature of the blocks should provide information about specific steps in cell membrane glycoprotein biosynthesis.

Five classes of Thy 1^- mutants have been identified by complementation analysis (2, 3). In somatic cell hybridization studies, mutants of each class complement mutants of all other classes but do not complement other mutants of the same class. The genetic studies are consistent with the hypothesis that one mutant class (class D) defines the structural gene coding for the Thy 1 alloantigenic determinant. The four remaining classes (A, B, C, and E) define genes other than that coding for the Thy 1 determinant but whose action is necessary for expression of Thy 1 on the cell surface.

The Thy 1 alloantigenic determinant is borne on a glycoprotein of approximate mol wt 25–30,000 (4) which, in the mouse, has been termed T25 (5, 6). Mutants of classes A, B, C, and E synthesize T25 molecules which are structurally different from the wild-type glycoprotein and which are not expressed on the cell surface, as determined by both biochemical and serological criteria (2, 7).[1] Pulse-chase experiments showed that the mutant molecules are degraded faster than the wild-type molecules;[1] however, the increased rate of degradation of the mutant molecules does not seem sufficient to account for the 500–1,000-fold deficit of T25 on the cell surface.

The existence of separate mutant classes, each of which synthesizes a mutant T25 molecule which does not reach the cell surface, raises questions about the

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* Supported by a Cancer Core grant no. CA-14195 to the Salk Institute.

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[1] I. Trowbridge, R. Hyman, and C. Mazauskas. *Cell*. In press.

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localization of T25 in mutant vs. wild-type cells and among the different mutant classes themselves. The T25 glycoprotein has been isolated from cultured mouse lymphoma cells and has been used to prepare a rabbit antiserum which specifically reacts with T25. This antiserum has allowed us to use immunohistochemical methods to detect T25 in wild-type and mutant cells. In the present study we have used immunofluorescence and immunoelectron microscopy to verify the absence of T25 from the cell membrane of mutant cells and, in preliminary experiments, to demonstrate directly the presence of T25 molecules within mutant cells.

Materials and Methods

Cell Lines. The wild-type AKR/J lymphoma line BW5147 (Thy 1^+) and the class A mutant BW5147 (Thy 1^-a) have been described previously (3, 7). (This mutant cell line was referred to as BW5147 (Thy 1^-) in previous publications). The cells were grown in suspension culture in Dulbecco's modified Eagle's medium with 10% horse serum (8).

Rabbit Anti-T25. Rabbit antiserum against T25 glycoprotein was prepared by repeated subcutaneous injections of the purified glycoprotein in complete Freund's adjuvant. A detailed account of the purification of the antigen and the properties of the antiserum will be given elsewhere. Briefly, T25 glycoprotein was purified from BW5147 (Thy 1^+) cells by solubilization of a crude membrane pellet with sodium deoxycholate, fractionation on a pea lectin-Sepharose column followed by gel filtration (9). The antiserum against T25 glycoprotein was cytotoxic for Thy 1^+ lymphomas but not Thy 1^- mutant cells. One major radioactive species with a 25,000 mol wt and corresponding to T25, was specifically precipitated by the antiserum from BW5147 (Thy 1^+) cells labeled either by the lactoperoxidase surface-labeling technique or metabolically labeled with [^{3}H]mannose or [^{3}H]leucine.

To demonstrate that intracellular staining was specific for T25, the rabbit anti-T25 antiserum was absorbed with intact BW5147 (Thy 1^+) cells to remove the anti-T25 antibodies, the remaining serum being used as a control. A pellet of 2×10^8 viable cells was incubated with 0.25 ml of a 1:10 dilution of the antiserum in phosphate-buffered saline for 30 min at room temperature. The cells were then centrifuged into a pellet and the supernate removed. This process was repeated three times.

Immunolabeling Reagents. For immunofluorescence staining, an indirect procedure was used, employing affinity purified goat antibodies to rabbit IgG. The goat antibodies were conjugated with fluorescein isothiocyanate or with lissamine rhodamine B sulfonyl chloride (10). For indirect immunoferritin staining, the goat antibodies were conjugated with six times recrystallized ferritin by the procedure of Kishida et al. (11).

Surface Immunofluorescent Labeling of T25 Protein. Both the wild-type and mutant cells were first treated in suspension with rabbit antisera to T25 protein in phosphate-buffered saline containing 0.2% bovine serum albumin, pH 7.4 for 30 min at 0°C. After washing, the cells were then reacted with fluorescein-conjugated goat antibodies to rabbit IgG for 30 min at 0°C. After washing the cells free of unbound protein, they were then fixed with 2% formaldehyde in 0.1 M phosphate buffer, pH 7.4, for 30 min at 0°C, and examined by fluorescent and Nomarski optics.

Intracellular Immunofluorescent Labeling of T25 Protein. Wild-type and mutant cells were fixed with 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 for 45 min at 0°C. Fixed cells were then kept overnight in 0.1 M phosphate buffer at 4°C, after which they were infused in 1.2 M sucrose, frozen, and sectioned in the frozen state. For immunofluorescent staining, such sectioning was carried out at about −30°C to section thicknesses of about 0.2 μm, whereas for immunoferritin staining, sections were cut at −80°C to thicknesses of approximately 700 Å (12–14). Both types of sections, after thawing, were then stained with rabbit antisera to T25. As controls, some sections were treated with normal rabbit IgG. The thicker sections were then treated with rhodamine-conjugated goat antibodies to rabbit IgG and processed for fluorescence observations. (Rhodamine conjugates were used here rather than fluorescein conjugates because of the autofluorescene of the glutaraldehyde-treated cells.) The thinner sections, on electron microscope grids, were labeled with ferritin-conjugated goat antibodies to rabbit IgG (13), and observed by electron microscopy.

Microscopy. Fluorescence observations were made with Zeiss Photoscope III by using a ×63 oil immersion lens and an epi-illuminator. Fluorescein and rhodamine fluorescences were excited with an Osram HBO 50 W bulb, and the filter combinations CZ 487710 and CZ 487714, respectively, were used for observations. Photography was performed with Kodak Plus X film.

Electron microscopic observations were made with a Phillips Model 300 electron microscope at 60 kV.

Results and Discussion

In this study we have used immunofluorescent and immunoelectron microscopic methods to directly visualize T25 in wild-type and Thy 1^- mutant cells. The results confirm the interpretation based on serological and biochemical methods. The wild-type cell line BW5147 (Thy 1^+) when stained in the living state by indirect immunofluorescence, by using rabbit anti-T25 as the primary reagent, shows T25 clustered over the cell surface (Figs. 1a and b). No surface fluorescence is seen when the class A mutant cell line BW5147 (Thy 1^-a) is stained in a similar manner (Figs. 2a and b). In frozen sections examined by immunofluorescence, the wild-type cell line shows staining predominantly at the cell membrane with areas of weak diffuse cytoplasmic fluorescence (Fig. 3). In contrast, the mutant cell line shows only diffuse patchy fluorescence throughout the cytoplasm (Fig. 4). This cytoplasmic staining was absent when the anti-T25 antiserum was first absorbed with intact Thy 1^+ cells (data not shown). Therefore there was no evidence that the anti-T25 serum contained antibodies which recognized contaminating intracellular specificities. Controls using normal rabbit serum in place of the antibodies to T25 were always negative.

A similar distribution of T25 was seen by immunofluorescence in mutants of classes C and E and their corresponding wild-type lines (data not shown).

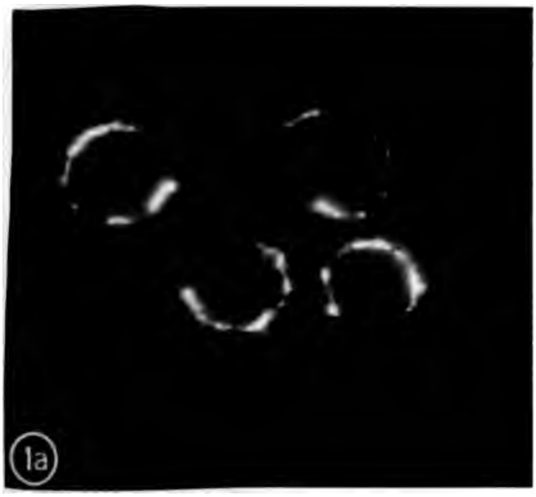
The development of ultrathin frozen sectioning methods of specimen preparation for immunoferritin electron microscopy (12, 13) has allowed us to carry out preliminary experiments at the ultrastructural level to study the distribution of T25 within the cytoplasm as well as on the surfaces of these cells. With the wild-type cells, the immunoferritin staining for T25 is mostly on the surface, with only light staining in the cytoplasm (Fig. 5). In the mutant cell line, little T25 is seen near the cell surface, but substantial amounts are found throughout the cytoplasm (Fig. 6). These results are therefore entirely consistent with the immunofluorescent results describod. Beyond that, however, it is of interest that the immunoferritin staining is often observed in clusters (Fig. 6, arrows) extending up to the cytoplasmic face of the surface membrane. This suggests a partially vesicular distribution of T25 protein inside the mutant cells, possibly extending beyond the endoplasmic reticulum up to the plasma membrane.

FIG. 1. a) Indirect fluorescein immunofluorescence staining of the T25 glycoprotein on the surfaces of intact BW5147 (Thy 1^+) cells, and b) the same field viewed with Nomarski optics.

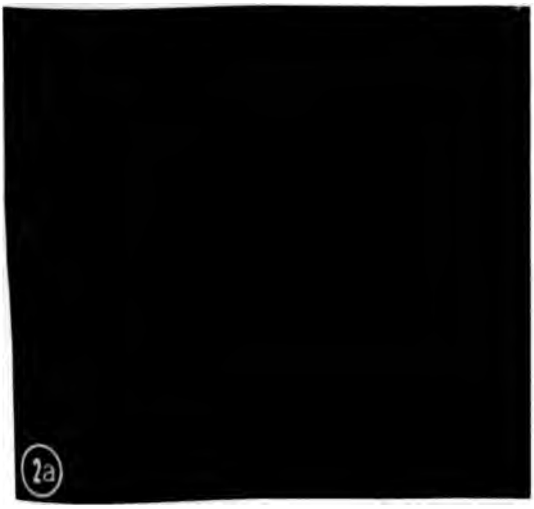
FIG. 2. a) The absence of surface staining for the T25 glycoprotein on intact BW5147 (Thy 1^-a) cells, and b) the same field viewed with Nomarski optics.

FIG. 3. Fixed frozen sections of BW5147 (Thy 1^+) cells stained by indirect rhodamine immunofluorescence for the T25 glycoprotein. Note the largely surface staining of the cells.

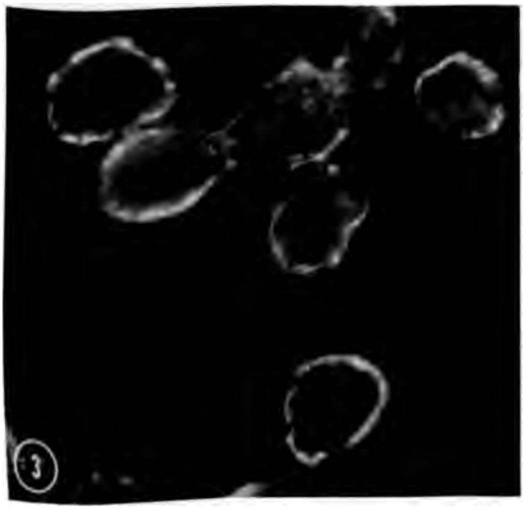
FIG. 4. Fixed frozen sections of BW5147 (Thy 1^-a) cells stained by indirect rhodamine immunofluorescence for the T25 glycoprotein. Note that most of the staining is cytoplasmic.


1a

1b


2a

2b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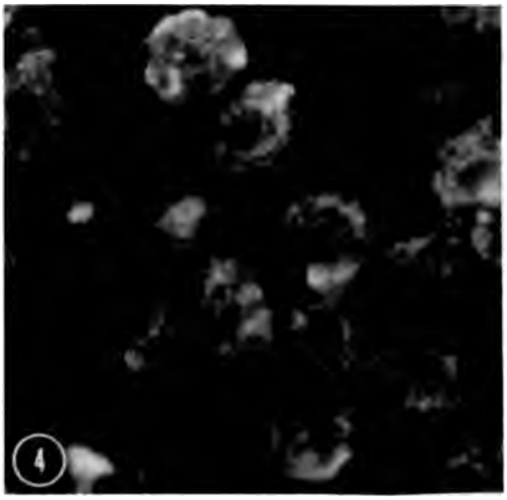
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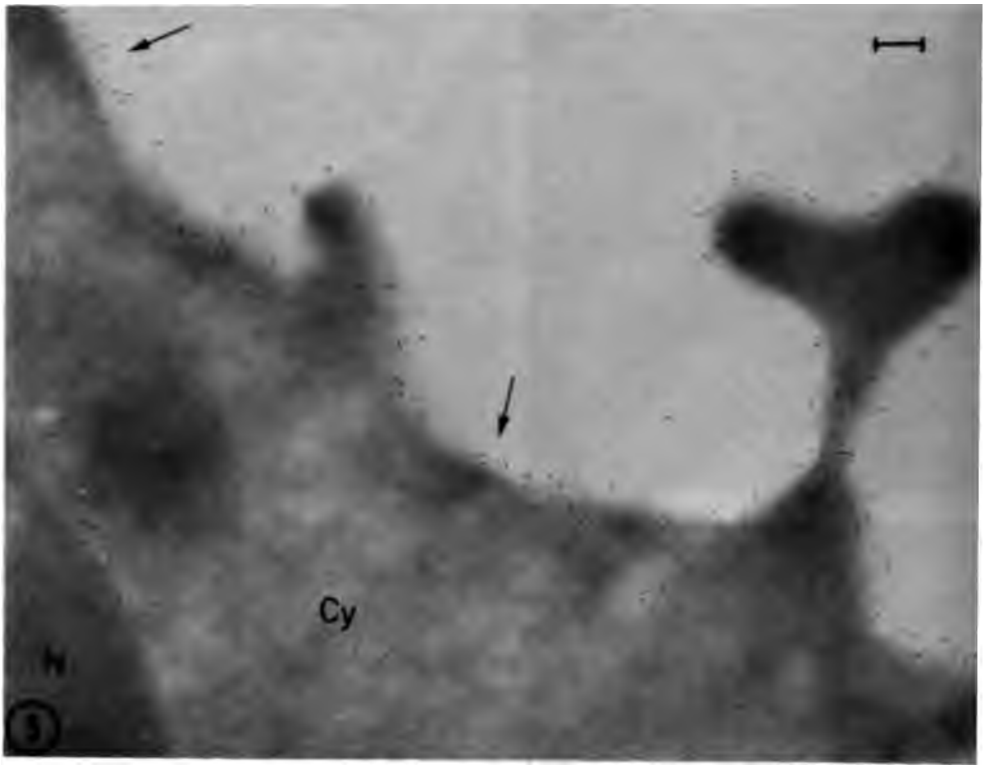
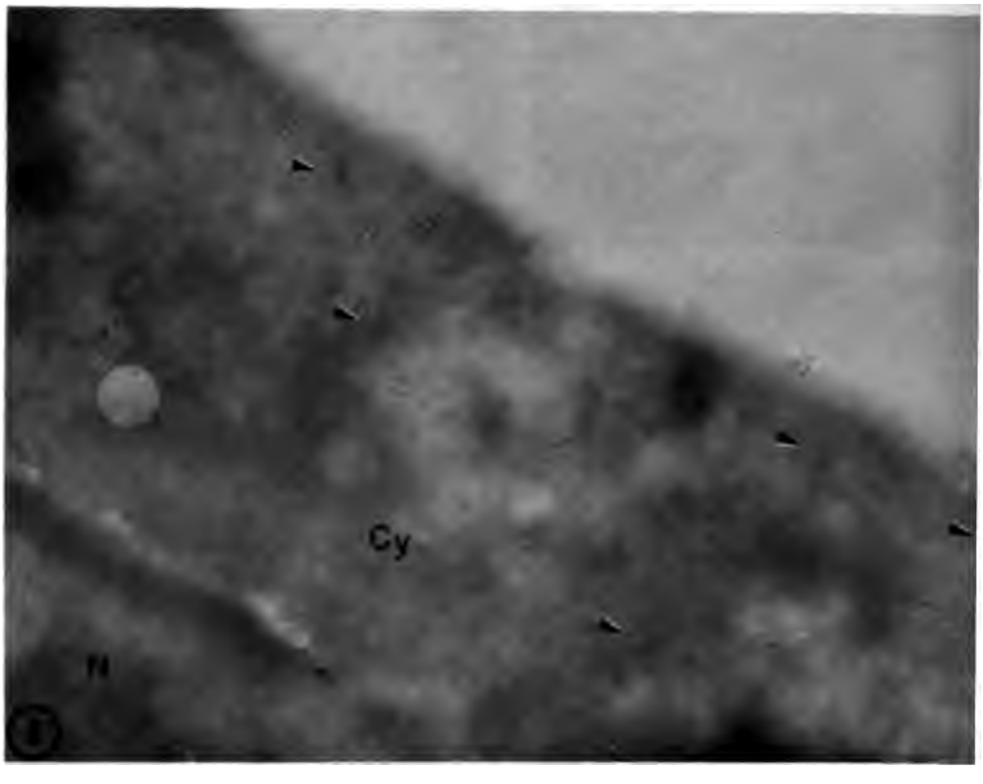


⑤



⑥

The genetic behavior of the class A, B, C, and E mutants and the fact that each mutant of these classes synthesizes an incomplete T25 molecule suggested the hypothesis that the mutants had defects in posttranslational steps of biosynthesis. Since T25 is a glycoprotein, the most likely block seemed to be a defect in carbohydrate biosynthesis (2, 7). Evidence supporting this idea comes from two experimental approaches. First, biochemical studies on the radiolabeled glycopeptides of mutant and wild-type T25 glycoproteins show that the oligosaccharides of the mutant molecules differ from those of the wild-type molecules.[1] Second, mutants with a Thy 1^- phenotype and which complement with class E mutants have been selected by prolonged exposure of mutagenized wild-type cells to cytotoxic concentrations of concanavalin A (I. Trowbridge, and R. Hyman, unpublished results). The class E mutants show a pleiotropic defect which affects the glycosylation of many glycoproteins, although only T25 is absent from the cell surface.[1]

Hickman et al. (15) have shown that IgA and IgE-secreting murine myeloma cells treated with tunicamycin, an inhibitor of glycosylation, show greatly reduced secretion of myeloma protein. By immunofluorescence unglycosylated protein is found inside the cells where it is present in a punctate distribution. Vesicles formed by the endoplasmic reticulum are seen in the tunicamycin-treated cells, and it was suggested that the unglycosylated myeloma protein is present mainly in these vesicles, but no immunoelectron microscopic observations were made.

To gain further information from immunoelectron microscopy on the specific nature of the blocks in different classes of Thy 1^- mutant it will be necessary to use newly developed methods for positive staining of frozen sections[2] to delineate better the internal membranes and ultrastructure of the cell. Furthermore, the use of synchronized cell cultures may permit the intracellular pathway of T25 biosynthesis to be elucidated.

Summary

The wild-type BW5147 (Thy 1^+) cell line and its Thy 1^- mutant derivative BW5147 (Thy 1^-a) were examined by immunofluorescence and immunoelectron microscopy for the presence of T25, the glycoprotein which bears the Thy 1 alloantigen. The wild-type cell had T25 predominantly localized on the cell surface. In the mutant cell line, T25 accumulated intracellularly and was present in a clustered distribution throughout the cytoplasm. T25 was not present on the surface of the mutant cell line in significant amount.

Received for publication 19 January 1978.

FIG. 5. Immunoferritin staining for the T25 glycoprotein on fixed, ultrathin frozen sections of BW5147 (Thy 1^+) cells. Note the largely surface localization of the ferritin stain.

FIG. 6. Immunoferritin staining for the T25 glycoprotein on fixed, ultrathin frozen sections of BW5147 (Thy 1^-a) cells. The ferritin stain is largely cytoplasmic and often seen in small clusters, up to the plasma membrane.

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[2] K. Tokuyasu. Manuscript submitted for publication.

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DIFFERENTIAL EFFECTS OF POLYADENYLIC:POLYURIDYLIC ACID AND LIPOPOLYSACCHARIDE ON THE GENERATION OF CYTOTOXIC T LYMPHOCYTES*

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The generation of cytotoxic T lymphocytes (CTLs)[1] in mixed leukocyte culture (MLC) reactions involves the interaction between different subpopulations of T cells (1). Precursors of CTLs (pre-CTL) respond primarily to antigens controlled by the *H-2K* and *H-2D* regions and develop into specific CTLs; these CTLs recognize the sensitizing *H-2K* and *H-2D* region-associated cytotoxic determinant (CD) antigens on target cells in the cell-mediated lympholysis (CML) assay (1–3). Another population of T cells responds to *H-2I* region-associated lymphocyte-defined determinant(s) (LD) with active proliferation and provides a helper effect in the development of CTLs (1–4). Thus, when responding and stimulating cells differ for both *H-2I* and *H-2K* or *H-2D* regions, there are strong proliferative and cytotoxic responses. If they differ only for the *H-2K* or the *H-2D* region, a low proliferative response and a weaker cytotoxic response are generated presumably due to weak LD-like activity associated with the *H-2K* and *H-2D* regions (5). When ultraviolet (UV) light-treated stimulating cells are used, there is no measurable proliferative or cytotoxic response even though the UV light-treated stimulating cells have the capacity to present the CD antigen. This is thought to be the result of the abolition of LD-like activity of the stimulating cells by UV light treatment and the lack of helper effect in the absence of the LD stimulus (6).

One approach to a study of the mechanisms underlying cell interactions involved in the generation of CTLs is to use agents that can modify or substitute for the helper effect. We now report the finding that the double stranded RNA, polyadenylic acid (poly A):polyuridylic acid (poly U) (poly A:U) and bacterial lipopolysaccharide W *Escherichia coli* 0127:B8 (LPS) can augment significantly the generation of CTLs in responder-stimulator cell combinations which differ for either the *H-2K* or the *H-2D* region. Two lines of evidence suggest that these two agents exert their effect at different steps in the development of the

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* Supported by National Institutes of Health grants CA-16836 and AI-08439. This is paper no. 139 from the Immunobiology Research Center, the University of Wisconsin, Madison, Wis., 53706.

[1] *Abbreviations used in this paper:* ADCC, antibody-dependent cellular cytotoxicity; BAEE, benzoyl-L-arginine ethyl ester; CD, cytotoxic determinant; CML, cell-mediated lympholysis; CRBC, chicken red blood cells; CTL, cytotoxic T lymphocytes; IgG, immunoglobulin G; LD, lymphocyte-defined determinant; LPS, lipopolysaccharide W *Escherichia coli* 0127:B8; MLC, mixed leukocyte culture; poly A:U, polyadenylic acid:polyuridylic acid; UV, ultraviolet.

cytotoxic response: (*a*) the effect of poly A:U depends on the presence of adherent cells, whereas the effect of LPS is independent of the presence of adherent cells, and (*b*) LPS promotes the development of CTLs when UV light-treated stimulating cells are used in the MLC whereas poly A:U does not.

Materials and Methods

Mice. The following mouse strains were used in this study. The letters in parentheses refer to the genotype derivation of the different regions of *H-2*, including *K*, *I*, *S*, and *D*: AQR (qkdd), B10.A (kkdd), B10.T(6R) (qqqd), B10.A(1R) (kkdb), B10.S (ssss), and C57BL/10 (bbbb).

Antiserum. BALB/c hyperimmune antiserum against chicken red blood cells (CRBC; 7) was a gift from Dr. Sylvia B. Pollack, Fred Hutchinson Cancer Research Center, Seattle, Wash.

In Vitro Sensitization and CML Assay. Generation of cytotoxic effector lymphocytes and CML assays were done according to the method of Peck and Bach (8). Stimulating cells were either mitomycin C-treated (indicated by the subscript m) or X-irradiated (indicated by the subscript x). The cells were cultured for 5 days and then the cytotoxicity determined by the CML assay. Percent cytotoxicity is expressed as the mean of four determinations ± SD.

Treatment with Poly A:U and LPS. Poly A:U (Miles Laboratories, Inc., Elkhart, Ind.) and LPS (lipopolysaccharide W *Escherichia coli* 0127:B8 obtained from Difco Laboratories, Detroit, Mich.) were added at the concentrations of 20 and 5 μg/ml, respectively, at the start of the culture.

UV Light Treatment of the Sensitizing Cells. To eliminate weak LD-like differences associated with *H-2K* and *H-2D* regions, sensitizing cells were UV irradiated according to the method of Lafferty et al. (9) with modification as described by Kuperman and Bach (10).

Antibody-Dependent Cellular Cytotoxicity (ADCC) Assay. The ADCC assay was carried out essentially as described by Pollack (11). Except where mentioned otherwise, the assay was done in Linbro microtitre plates (Linbro Chemical Co., Hamden, Conn.) with 2×10^4 antibody-coated CRBC targets per well in a final volume of 0.2 ml.

Trypsin Treatment of Effector Cells. Effector cells from MLCs and normal spleen cells were treated with 0.25% trypsin (twice crystallized, 10,000 benzoyl-L-arginine ethyl ester (BAEE) U/mg, obtained from Grand Island Biological Co., Grand Island, N.Y.) in Hanks' balanced salt solution containing $NaHCO_3$ for 45 min at 37°C, and afterwards the cells were washed twice.

Preparation of Aggregated Immunoglobulin G. Human immunoglobulin G (IgG), obtained from Miles Laboratories, was heat-aggregated by incubation at 63°C for 15 min at a concentration of 20 mg/ml in phosphate-buffered saline, pH 7.4.

Results

Enhancement of CTL Generation by Poly A:U and LPS. We have observed that both poly A:U and LPS enhance the generation of CTLs in several responder-stimulator combinations which differ for both the *H-2I* and the *H-2K* or *H-2D* regions or for the entire *H-2* complex (data not shown). In such combinations there is generally a strong proliferative response and marked generation of CTLs even in the absence of poly A:U and LPS. In contrast, in combinations that differ for the *H-2K* or *H-2D* region only, both responses are generally very low.

Table I shows the effect of poly A:U and LPS on the generation of CTLs in such combinations using the responder-stimulator pairs AQR-B10.A and B10.A(1R)-B10.A which differ for *H-2K* and *H-2D*, respectively. The presence of LPS or poly A:U in the culture enhances the generation of CTLs in both the combinations. The optimum concentrations are 5 μg/ml for LPS and 20 μg/ml for poly A:U. LPS and poly A:U cause enhancement in the AQR-B10.A combination to a similar degree consistently, although the magnitude of the enhancement varies among experiments. In the B10.A(1R)-B10.A combination,

TABLE I

Effect of Poly A:U and LPS in the Generation of CTLs in Responder-Stimulator Combinations Differing for H-2K Region and H-2D Region

Responder + stimulator combination	Additions	Cytotoxicity ± SD on the targets		
		B10.A (kkdd)	AQR (qkdd)	C57BL/10 (bbbb)
			%	
I AQR + B10.A$_m$	None	4.8 ± 3.1	4.3 ± 3.0	–
AQR + B10.A$_m$	LPS, 5 μg/ml	30.7 ± 4.2	4.1 ± 3.6	–
AQR + B10.A$_m$	Poly A:U, 20 μg/ml	34.2 ± 8.5	6.4 ± 3.4	–
II B10.A(1R) + B10.A(1R)$_x$	None	−1.9 ± 7.6	–	–
B10.A(1R) + B10.A(1R)$_x$	LPS, 5 μg/ml	−5.0 ± 7.8	–	–
B10.A(1R) + B10.A(1R)$_x$	Poly A:U, 20 μg/ml	2.0 ± 10.0	–	–
B10.A(1R) + B10.A$_x$	None	11.6 ± 5.0	–	–
B10.A(1R) + B10.A$_x$	LPS, 5 μg/ml	36.8 ± 9.4	–	–
B10.A(1R) + B10.A$_x$	Poly A:U, 20 μg/ml	40.8 ± 3.0	–	–
III B10.A(1R) + B10.A$_x$	None	15.0 ± 6.8	–	1.7 ± 5.7
B10.A(1R) + B10.A$_x$	Poly A:U, 20 μg/ml	44.2 ± 2.6	–	−8.9 ± 9.6

Effector:target ratios were 100:1 in I, 50:1 in II, and 40:1 in III.

both LPS and poly A:U caused similar enhancement in some experiments, but in others LPS caused enhancement to a greater degree than poly A:U.

The results shown in Table I were obtained at a single effector:target ratio for each experiment. However, the cytotoxic responses generated both in the presence and absence of LPS and poly A:U were measured at different effector:target ratios, and in all cases percent cytotoxicity was related linearly to the logarithm of effector:target ratio (data not shown) characteristic of T cell-mediated cytotoxicity (12).

The enhanced cytotoxicity observed is the result of enhanced generation of CTLs and not of the presence of poly A:U or LPS during the cytotoxicity assay as the addition of these agents during the CML assay does not affect cytotoxocity (data not shown). The CTLs generated in the presence of LPS and poly A:U are specific for the sensitizing antigen. In the B10.A(1R)-B10.A combination, the CTLs generated in the presence of poly A:U are cytotoxic for B10.A, whereas they are not or only minimally cytotoxic for C57BL/6 (bbbb) which does not share the sensitizing antigen, H-2D^d (Table I). The CTLs are cytotoxic for B10.T(6R) (qqqd) which shares the sensitizing antigen and not cytotoxic for another nonspecific target B10.S (ssss) (results not shown). Similar results were also obtained with the CTLs generated in the presence of LPS. Furthermore, specific CTLs are also generated in the AQR-B10.A combination in the presence of these agents (data not shown).

Because LPS and poly A:U are known to act as adjuvants in antibody response (13–16), experiments were carried out to test whether there was any contribution by ADCC to the enhancement of cytotoxic response observed. AQR spleen cells were stimulated with X-irradiated B10.A spleen cells in the

TABLE II

Effect of Treatment with Trypsin or Aggregated IgG on the Cytotoxic Cells

Effector cells	Treatment	Effector: target ratio	Cytotoxicity ± SD	
			CML assay using B10.A targets	ADCC assay using CRBC targets
			%	%
AQR + B10.A_x	None	50:1	25.6 ± 2.8	11.6 ± 3.9
AQR + B10.A_x	Trypsin	50:1	−5.0 ± 1.9	–
AQR + B10.A_x	Aggregated IgG	50:1	30.1 ± 2.2	–
AQR + B10.A_x + LPS	None	50:1	78.0 ± 3.9	5.4 ± 3.5
AQR + B10.A_x + LPS	Trypsin	50:1	7.6 ± 8.5	–
AQR + B10.A_x + LPS	Aggregated IgG	50:1	63.5 ± 17.2	–
AQR + B10.A_x + poly A:U	None	50:1	71.6 ± 9.8	6.7 ± 1.2
AQR + B10.A_x + poly A:U	Trypsin	50:1	25.1 ± 8.3	–
AQR + B10.A_x + poly A:U	Aggregated IgG	50:1	72.3 ± 9.7	–
Normal AQR spleen cells	None	50:1	–	55.0 ± 8.0
Normal AQR spleen cells	None	12.5:1	–	28.4 ± 4.6
Normal AQR spleen cells	Trypsin	12.5:1	–	26.9 ± 4.0
Normal AQR spleen cells	Aggregated IgG	50:1	–	7.8 ± 4.0

Effector cells were generated in MLC in the presence and absence of LPS or poly A:U as indicated except in the case of normal spleen cells. Aggregated IgG was added to the assay mixture at a concentration of 4 mg/ml. 1:5 dilution of the anti-CRBC antiserum was used in the ADCC assay.

presence and absence of LPS or poly A:U, and with the resulting cells CML assay using B10.A target cells and ADCC assay using CRBC-anti-CRBC system were carried out. As shown in Table II both LPS and poly A:U enhanced the cytotoxicity in CML but did not enhance the cytotoxicity in ADCC. The effector cell in ADCC is trypsin resistant (17, 18), whereas the cytotoxic T lymphocyte is sensitive to trypsin treatment (18). The data presented in Table II show that the cytotoxic effector cells generated in the presence of LPS and poly A:U are sensitive to trypsin treatment. Under the same experimental conditions, normal spleen cell population which participates in ADCC is trypsin resistant (Table II). Furthermore, aggregated IgG, which is known to inhibit ADCC (18), does not inhibit the cytotoxicity by the cells generated in cultures containing LPS or poly A:U. All these results clearly indicate that there is no measurable contribution by ADCC to the cytotoxic responses generated in the presence of LPS and poly A:U.

Table III shows the effect of poly A:U and LPS on the generation of CTL in the presence and absence of adherent cells. In the absence of adherent cells poly A:U does not enhance the generation of CTLs. LPS, on the other hand, enhances CTL generation both in the presence and absence of adherent cells.

In the two responder-stimulator combinations described above, there is a weak proliferative response and a weak cytotoxic response when X-irradiated stimulating cells are used. This is thought to be due to weak "LD-like" differences associated with the *H-2K* and *H-2D* regions as described elsewhere (5). The weak LD stimulus and the weak proliferative response can be abolished by using UV light-treated stimulating cells which do, however, present CD

TABLE III

Effect of Adherent Cell Removal on the Action of Poly A:U and LPS

Responder + stimulator combination	Additions	Cytotoxicity ± SD on B10.A target cells	
		+ Adherent cells	− Adherent cells
		%	
B10.A(1R) + B10.A$_x$	None	9.3 ± 4.3	1.3 ± 2.6
B10.A(1R) + B10.A$_x$	Poly A:U, 20 μg/ml	33.1 ± 1.8	3.0 ± 2.6
B10.A(1R) + B10.A$_x$	LPS, 5 μg/ml	61.9 ± 3.0	55.9 ± 3.9

Spleen cells, 30×10^6, in 4 ml of medium were taken in 60 × 15-mm Falcon tissue culture dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) and incubated for 90 min at 37°C in an atmosphere of 95% air and 5% CO$_2$. The nonadherent cells were transferred to a fresh dish, and the incubation was continued for an additional 90 min. The nonadherent cells were harvested and used in the MLC reaction. Nonadherent responder and stimulator cells were prepared separately. Effector:target ratio was 40:1.

TABLE IV

Effect of Poly A:U and LPS in the Presence of UV Light-Treated Stimulating Cells

Responder + stimulator combination	Additions	Cytotoxicity ± SD on the targets	
		B10.A (kkdd)	B10.T(6R) (qqqd)
		%	
AQR + AQR$_x$	None	−4.7 ± 2.0	−3.1 ± 2.9
AQR + B10.A$_x$	None	5.6 ± 1.0	−1.8 ± 1.6
AQR + B10.A$_{uv}$	None	−8.5 ± 3.0	−2.3 ± 3.0
AQR + B10.A$_{uv}$	Poly A:U, 20 μg/ml	−3.6 ± 3.8	−1.7 ± 1.5
AQR + B10.A$_{uv}$	LPS, 5 μg/ml	23.3 ± 3.1	−2.3 ± 1.5

Effector:target ratio in the cytotoxicity assay was 70:1.

antigens to the responding cells (6). The effects of poly A:U and LPS on the generation of CTLs in the absence of the weak LD-like stimulus was thus tested using UV light-treated stimulating cells. As shown in Table IV poly A:U does not enhance the generation of CTLs whereas LPS retains the enhancing effect when UV light-treated stimulating cells are used.

Discussion

Our observation that both LPS and poly A:U enhance the generation of CTLs in responder-stimulator combinations that differ for both the *H-2I* and the *H-2K* or *H-2D* regions or for the entire *H-2* complex is similar to the finding of Wagner and Cone (19). These investigators found that poly A:U amplified the generation of CTLs in in vitro response of CBA (*H-2*^{k}) cells against mitomycin-treated BALB/c (*H-2*^{d}) cells; they did not test the effect of LPS in their system. In these responder-stimulator combinations there is generally a strong proliferative response and marked generation of CTLs even in the absence of LPS and poly A:U. In contrast, a responder-stimulator combination that differs for the *H-2K* or *H-2D* region shows low level proliferative and cytotoxic responses (5). In such combinations both poly A:U and LPS enhance the generation of cytotoxic cells significantly. This augmentation in cytotoxicity is due to an increased generation of CTLs and not to increased ADCC (Table II). The CTLs generated in the presence of these agents are specific for the sensitizing antigen.

In the presence of poly A:U, the incorporation of [^{3}H]thymidine by the cells in the MLC is enhanced two- to threefold. LPS, on the other hand, enhances [^{3}H]thymidine incorporation 8- to 10-fold (results not shown). It is not clear whether this effect on proliferative response has a role in the enhanced generation of CTL.

When UV light-treated stimulating cells are used, neither the proliferative response nor CTL generation are detectable, as previously described (6), presumably due to abolition of LD-like activity on the stimulating cells by UV light irradiation and the resulting lack of initiation of any response. In the presence of LPS, however, a cytotoxic response is generated against the CD antigens on the UV light-treated stimulating cells. On the other hand, no cytotoxic response is generated against UV light-treated stimulating cells in the presence of poly A:U. These results are consistent with the notion that LPS but not poly A:U has the capacity to exert appropriate signals and promote the initiation of cytotoxic response in the presence of UV light-treated stimulating cells and in the absence of any LD or LD-like activity. An alternate explanation is that LPS acts on UV light-treated stimulating cells and allows the expression of LD-like activity. But this possibility is less likely as UV light treatment has a deleterious effect on the metabolic activity of the cells.

Both poly A:U and LPS act as adjuvants in antibody response to T-dependent antigens (13–16), and they are thought to exert the adjuvant effect through helper T cells (20–22). It is conceivable that the enhancement of CTL generation caused by LPS and poly A:U in an MLC is also due to their effect on helper T cells.

The present results suggest that LPS and poly A:U enhance cytotoxic responses by different mechanisms. Poly A:U may only be able to amplify an ongoing cytotoxic response, whereas LPS may have the capacity to promote the initiation of cytotoxic response and also amplify the response. Because both the agents are potent at expanding an ongoing response, the inability of poly A:U to help initiate a response suggests that the signals for initiation and expansion of the cytotoxic response may be different. That these two agents exert their effect at two different steps in the development of CTLs is also supported by the finding that poly A:U requires the presence of adherent cells for its action whereas LPS does not under the in vitro conditions used.

Summary

In a mixed leukocyte culture (MLC) reaction of allogenic mouse spleen cells differing for *H-2K* or *H-2D*, only a weak cytotoxic response is generated. This cytotoxic response is augmented significantly if bacterial lipopolysaccharide (LPS), 5 μg/ml, or polyadenylic acid (poly A):polyuridylic acid (poly U), 20 μg/ml, is present in the culture. The cytotoxic cells generated in the presence of

these two agents are specific for the sensitizing H-2K or H-2D antigen. Two lines of evidence suggest that these two agents exert their effect at different steps in the development of cytotoxic lymphocytes: (*a*) the effect of poly A:U depends on the presence of adherent cells, whereas the effect of LPS is independent of the presence of adherent cells and (*b*) LPS promotes the development of cytotoxic cells when ultraviolet light-treated stimulating cells are used in the MLC whereas poly A:U does not.

We thank Dr. Sylvia B. Pollack, Fred Hutchinson Cancer Research Center, Seattle, Wash., for her generous gift of anti-CRBC antiserum. We also thank Prof. Fitz H. Bach for his support, encouragement, and help in the preparation of this manuscript.

Received for publication 6 September 1978.

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LUNG TUMOR-ASSOCIATED DEREPRESSED ALLOANTIGEN CODED FOR BY THE *K* REGION OF THE *H-2* MAJOR HISTOCOMPATIBILITY COMPLEX

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According to the concept of immune surveillance, a major function of the immune system is the detection and elimination of nascent autochthonous tumors (1–3). There is considerable evidence that cellular immune responses evoked in mice by immunization with allogeneic cells (4–5) and with virally infected (6–10) or chemically treated (11) syngeneic cells are mainly directed against cell surface antigens coded for by the *K* and *D* regions of the *H-2* major histocompatibility complex (MHC).[1] Immune responses against nascent tumors might similarly involve recognition of altered or derepressed products coded for by the MHC (12, 13). This possibility is supported by ongoing studies on the tumor-associated transplantation antigen (TATA) expressed by transplacentally induced lung tumors of C3HfeB/HeN mice. As reported elsewhere (14, 15), some of these tumors grow poorly when inoculated into syngeneic mice but will grow readily if transplanted to (C3HfeB/HeN × A)F_1 mice. The preferential growth in the F_1 recipients occurs because the TATA responsible for syngeneic immunity is present as a normal tissue antigen in strain A mice and in its F_1 hybrids (14–16). This antigen is also expressed in normal tissues of C3H/HeN mice, the strain from which the C3HfeB/HeN strain was originally derived.[2] Genetic studies have indicated that, in strain A mice, the lung tumor-associated normal tissue alloantigen is coded for by a gene linked to the MHC (16). The antigen is not expressed by normal tissues of either C57BL/6 or DBA/2 mice although it can be demonstrated on a proportion of transplacentally induced lung tumors of these strains (17). These observations suggest that a genetic locus associated with the MHC is subject to regulation and that in mouse strains in which this locus is normally repressed it may code for a TATA on chemically induced lung tumors.

In the present study we have investigated the strain and tissue distribution of the lung tumor-associated alloantigen. The results clearly implicate the *K* region of the MHC in controlling the expression of the alloantigen. Expression of this *H-2K* region-coded alloantigen in normal tissues does not correlate directly with susceptibility to the spontaneous development of lung tumors in mice.

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[1] *Abbreviations used in this paper*: C3H, C3H/HeN strain mice; C3Hf, C3HfeB/HeN strain mice; MHC, major histocompatibility complex; SE, standard error of the mean; TATA, tumor-associated transplantation antigen.

[2] W. J. Martin, T. G. Gipson, M. A. Conliffe, W. G. Cotton, L. F. Dove, and J. M. Rice. Histocompatibility difference between C3HfeB/HeN and C3H/HeN mice. Manuscript submitted for publication.

Materials and Methods

Mice. Inbred strains of mice used in this study were obtained from the Animal Production Unit, National Institutes of Health, The Jackson Laboratory, Bar Harbor, Maine, Dr. Jack Stimpling, McLauglin Research Institute, Great Falls, Mont., Dr. David Sachs, National Cancer Institute, Bethesda, Md., and Dr. M. Zalenski of the State University of New York. A detailed description of the derivation of the C3HfeB/HeN strain from the C3H/HeN strain will be provided elsewhere.[2] These strains will be subsequently referred to as C3Hf and C3H, respectively.

Tumors. The lung tumor 85 was induced by the transplacental administration of the carcinogen 1-ethyl-1-nitrosourea to a 13-day-pregnant C3Hf mouse (0.5 μm/g maternal body wt). The tumor was detected in a 1-yr-old offspring of the treated mouse. Fragments of this tumor were transplanted into groups of C3Hf and (C3Hf × A)F_1 recipients. The tumor grew only in the (C3Hf × A)F_1 mice. The tumor grew progressively in these mice. The tumor also grew when transplanted to X-irradiated C3Hf mice. Lung tumor tissue derived from a tumor-inoculated X-irradiated C3Hf mouse was explanted to tissue culture. Tissue culture-derived cells readily form adenocarcinomas when transplanted into either (C3Hf × A)F_1 or C3H mice.

Antitumor Immunization. Tissue was removed from donor mice and cut into approximately 1-mm^3 fragments. Recipient mice were anesthetized and inoculated with two tissue fragments, one given subcutaneously and one given intraperitoneally.

X-irradiation. 2 wk after immunization the mice received 400 rads whole body X-irradiation at a dose rate of 125 rads/min. Control mice were similarly X-irradiated. 24 h after X-irradiation, the mice were challenged intradermally with 10^5 tissue culture-derived lung tumor 85 cells. Tumor growth was determined during the ensuing 21 days and recorded as mean tumor diameter ± standard error (SE).

Results

Growth Characteristics of Lung Tumor 85. We have previously shown that the C3Hf mouse-derived lung tumor 85 does not grow progressively when inoculated into normal C3Hf recipients but will grow progressively when inoculated into either (C3Hf × A)F_1 hybrid or C3H recipients[2] (14–16). The tumor will grow in sublethally X-irradiated C3Hf mice provided the mice were not specifically immunized against the lung tumor before X-irradiation. Fig. 1 depicts the characteristic growth of 10^5 lung tumor 85 cells inoculated intradermally into normal C3Hf and C3H recipients. In Fig. 2 the growth of the lung tumor in X-irradiated C3Hf mice preimmunized with lung tissue from either C3Hf, A, or C3H mice is illustrated. The tumor grows progressively in X-irradiated, previously untreated C3Hf mice and in C3Hf mice preimmunized with syngeneic lung tissue. Preimmunization with lung tissue from C3H or A mice protects X-irradiated C3Hf mice from subsequent lung tumor 85 challenge. We have used this system to test the tissue distribution and genetic origin of the antigen that induces radioresistant immunity in C3Hf mice against lung tumor 85.

Tissue Distribution of Tumor-Associated Alloantigen. C3Hf mice were immunized with either lung, liver, or kidney tissue of strain A mice and with spleen cells of (C3Hf × A)F_1 mice. The immunized mice were subsequently X-irradiated and challenged with 10^5 tumor 85 cells. The results recorded in Table I depict the mean diameter of tumors present in the mice 15 days after tumor inoculation. All the tissues of strain A mice tested were able to evoke radioresistant anti-lung tumor immunity in C3Hf mice. In other experiments it has been shown that these same tissues from C3H mice can similarly immunize C3Hf mice against tumor 85. In contrast, no tissue tested from C3Hf mice provided anti-tumor protection (Table I).

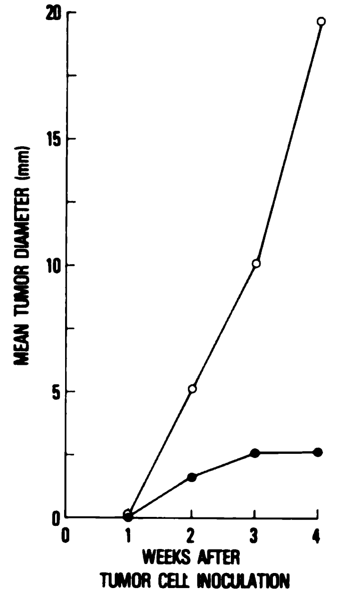

FIG. 1. Mean tumor diameter of lung tumor 85 inoculated intradermally into either C3H (○) or C3Hf (●) mice. 10 mice of each strain were used in this experiment. Each mouse received 10^5 lung tumor cells.

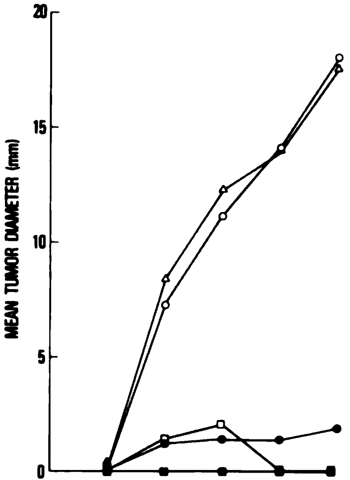

FIG. 2. Mean tumor diameter of lung tumor 85 inoculated intradermally into either normal C3Hf mice (●) or X-irradiated C3Hf mice either not preimmunized (○) or preimmunized 14 days before tumor challenge with lung tissue from C3Hf (△), C3H (□), or A (■) strain mice. Each mouse received 10^5 lung tumor cells. 10 mice were used per group.

Tumor-Associated Alloantigen on Embryo Tissue. Lung tumor 85 was induced by administering the carcinogen ethyl-nitrosourea during fetal development. Experiments were therefore performed to determine whether the tumor-associated alloantigen might be normally expressed during embryonic

TABLE I

Tissue Distribution of Lung Tumor 85 Cross-Reactive Alloantigen in A and C3H Strain Mice

Exp.	Tissue used for immunization	Donor strain	Tumor growth in X-irradiated C3Hf mice	
			Tumor incidence	Mean tumor diameter ± SE
				mm
1	None	–	8/8	6.4 ± 0.4*
	Lung	A	1/8	0.5 ± 0.5
	Liver	A	1/8	0.6 ± 0.6
	Kidney	A	1/7	0.4 ± 0.4
	Spleen	(C3Hf × A)F$_1$	0/7	0.0
2	None	–	11/11	11.2 ± 0.5
	Lung	C3Hf	7/7	12.2 ± 1.3
	Liver	C3Hf	6/6	9.9 ± 0.6
	Kidney	C3Hf	6/6	12.0 ± 1.2
	Spleen	C3Hf	6/6	11.5 ± 0.5
3	None	–	6/6	12.2 ± 1.4
	Embryo (15 day)	C3H	0/10	0.0
	Embryo (15 day)	C3Hf	10/10	13.4 ± 0.8
	Embryo (11 day)	C3Hf	6/6	13.9 ± 1.0
	Embryo (18 day)	C3Hf	5/6	10.2 ± 2.4

* Tumor growth in the experiment was recorded at day 15 after tumor challenge. In all other experiments the tumor growth was recorded at 21 days after tumor challenge.

development of C3Hf mice. C3Hf mice were immunized with tissue fragments from 15-day embryos derived from pregnant C3Hf or C3H mice. The immunized mice were X-irradiated and challenged with lung tumor 85 cells. The data in Table I (exp. 3) indicate that mice were protected by preimmunization with C3H embryo tissue, but not by C3Hf-derived embryo tissue. Tissue from 11- and 18-day C3Hf embryos similarly failed to confer effective anti-tumor immunity (Table I).

Strain Distribution of Tumor-Associated Alloantigen. C3Hf mice were immunized with normal liver tissue from a wide variety of mice of various MHC haplotypes. Mice that share the $H\text{-}2^a$ haplotype with strain A mice, or the $H\text{-}2^k$ haplotype with C3H mice, induced highly significant immunity in C3Hf mice (Table II). Liver tissue from CBA (532) strain mice ($H\text{-}2^{ka}$ haplotype) was similarly found to induce anti-lung tumor 85 immunity in C3Hf mice. Tissue from mice of other *H-2* haplotypes tested failed to induce significant immunity in C3Hf mice (Table III).

Linkage of Gene Coding for Alloantigen to K Region of MHC. As can be noted in Tables II and III, the alloantigen is expressed in liver tissue of the congenic strains B10.A (H-2^a) and B10.BR (H-2^k) but not in liver tissue of B10 congenic mice expressing other MHC haplotypes. Similarly, the gene is not expressed in C3H-derived mice whose MHC haplotype is different from $H\text{-}2^k$ (Table III, exp. 2). The gene coding for the expression of the alloantigen is therefore linked to the MHC. The MHC is known to comprise at least six clearly definable regions, designated *K, IA, IB, IC, S,* and *D*. The origin of the *K, IA,* and *IB* regions of $H\text{-}2^a$ and $H\text{-}2^k$ mice are similar and are designated *k*. The

TABLE II
Strain Distribution of Lung Tumor 85 Cross-Reactive Alloantigen

Donor strain of tissue used for immunization	MHC haplotype of donor strain	Tumor growth in X-irradiated C3Hf mice	
		Tumor incidence	Mean tumor diameter at 21 days ± SE
			mm
None	–	7/7	11.2 ± 0.9
C3Hf	–	6/6	12.2 ± 1.3
A	*a*	3/11	1.1 ± 0.6
AL	*a*	1/8	0.5 ± 0.5
B10.A	*a*	0/6	0.0
C3H	*k*	2/8	2.0 ± 1.3
CBA	*k*	1/8	0.7 ± 0.8
ST/b	*k*	0/8	0.0
RF	*k*	3/7	1.4 ± 0.6
AKR	*k*	0/8	0.0
C57BR	*k*	1/7	1.6 ± 1.7
B10.BR	*k*	3/7	2.8 ± 1.0
Ma/My	*k*	2/8	1.4 ± 0.9
CBA(M523)	*ka*	0/8	0.0

TABLE III
Strain Distribution of Lung Tumor 85 Cross-Reactive Alloantigen

Exp.	Donor strain of tissue used for immunization	MHC haplotype of donor strain	Tumor growth in X-irradiated C3Hf mice	
			Tumor incidence	Mean tumor diameter at 21 days ± SE
				mm
1	None	–	11/11	11.7 ± 0.5
	AKR	*k*	1/6	1.2 ± 1.3
	B10	*b*	6/6	11.2 ± 0.9
	B10.RIII	*r*	7/7	11.2 ± 0.5
	B10.PL	*u*	5/5	10.1 ± 0.4
	B10.P	*p*	6/6	12.8 ± 0.4
	B10.HTG	*g*	7/7	11.9 ± 0.5
	B10.WB	*ya*	7/7	11.5 ± 0.6
	B10.M	*f*	7/7	12.3 ± 0.7
	B10.Y	*pa*	6/6	11.2 ± 0.4
	B10.Q	*q*	7/7	11.3 ± 0.4
	B10.S	*s*	5/5	13.4 ± 0.6
	B10.SM	*v*	5/6	11.0 ± 2.3
	A.CA	*f*	6/6	11.9 ± 0.9
	DBA/2	*d*	5/5	11.1 ± 0.8
	LG	*df*	9/9	12.2 ± 0.4
	SJL	*s*	6/6	9.6 ± 0.2
	SWR	*q*	8/8	12.8 ± 0.5
2	None	–	8/8	9.6 ± 0.7
	C3H/He	*k*	1/7	1.4 ± 1.5
	C3H.NB	*p*	6/7	8.2 ± 2.3
	C3H.JK	*j*	8/9	9.1 ± 1.5

TABLE IV

Expression of Lung Tumor 85 Cross-Reactive Alloantigen in Congenic-Resistant Recombinant Strains of Mice

Exp.	Donor strain of tissue used for immunization	Origin of MHC regions of donor strain						Tumor growth in X-irradiated C3Hf mice	
		K	*IA*	*IB*	*IC*	*S*	*D*	Tumor incidence	Mean tumor diameter at 21 days ± SE
									mm
1	None			–				9/9	14.2 ± 0.7
	B10.A (1R)	*k*	*k*	*k*	*d*	*d*	*b*	0/7	0.0
	B10.A (2R)	*k*	*k*	*k*	*d*	*d*	*b*	0/7	0.0
	B10.A (3R)	*b*	*b*	*b*	*d*	*d*	*d*	11/11	12.1 ± 1.2
	B10.A (4R)	*k*	*k*	*b*	*b*	*b*	*b*	0/10	0.0
	B10.A (5R)	*b*	*b*	*b*	*d*	*d*	*d*	7/7	14.3 ± 0.9
	B10.A (15R)	*k*	*k*	*k*	*d*	*d*	*b*	0/6	0.0
	B10.A (18R)	*b*	*b*	*b*	*b*	*b*	*d*	7/7	12.9 ± 1.0
2	None			–				5/5	12.9 ± 0.2
	B10.AQR	*q*	*k*	*k*	*d*	*d*	*d*	7/7	11.6 ± 0.6
	A.TL	*s*	*k*	*k*	*k*	*k*	*d*	6/6	11.8 ± 0.7
	C3H.OH	*d*	*d*	*d*	*d*	*d*	*k*	8/8	11.4 ± 0.4
	B10.BR	*k*	*k*	*k*	*k*	*k*	*k*	3/7	1.5 ± 0.7

remaining regions (*IC*, *S*, and *D*) have a different origin and are designated *d* and *k* for $H\text{-}2^a$ and $H\text{-}2^k$ mice, respectively (18). (The MHC regions of $H\text{-}2^b$ mice, e.g. B10, are each designated *b*.) Because $H\text{-}2^a$ and $H\text{-}2^k$ mice share the alloantigen cross-reactive with the TATA of the tumor 85, the genetic locus coding for this antigen is presumably linked more closely to the *K*, *IA*, and *IB* regions of the MHC than to the *IC*, *S*, and *D* regions. To define more precisely this linkage to the MHC, C3Hf mice were immunized with tissue from a variety of recombinant mice (19) inheriting various regions of the $H\text{-}2^a$ and $H\text{-}2^k$ haplotypes. It is apparent from Table IV that tissue from 1R, 2R, 4R, and 15R mice conferred anti-tumor immunity. Mice preimmunized with tissue from the 3R, 5R, and 18R recombinants failed to achieve effective immunity to lung tumor challenge. Thus, the haplotypes common to all the strains conferring immunity are $H\text{-}2K^k$ and $H\text{-}2IA^k$. To further delineate the genetics of expression of the alloantigen, tissues were tested from two strains of mice that express the $H\text{-}2IA^k$ haplotype but not the $H\text{-}2K^k$ haplotype. As shown in Table IV (exp. 2) neither tissue from A.TL ($H\text{-}2K^s$) nor B10.AQR ($H\text{-}2K^q$) mice was capable of inducing anti-lung tumor immunity in C3Hf mice.

Tissues from each of the strains shown to induce radioresistant immunity in C3Hf mice were similarly tested for their ability to induce radioresistant immunity against the lung tumor 85 in C3H mice. As shown in Table V neither tissues from the congenic resistant recombinant strains nor tissues from representative $H\text{-}2^k$ or $H\text{-}2^a$ haplotype strains conferred effective anti-tumor immunity in C3H mice.

TABLE V

Inability to Induce Radioresistant Anti-Lung Tumor 85 Immunity in C3H Mice Using Tissue from Mice that Express the Lung Tumor-Associated Cross-Reactive Alloantigen

Exp.	Donor strain used to immunize C3H mice	Origin of MHC regions of donor strains						Tumor growth in X-irradiated C3H mice	
		K	*IA*	*IB*	*IC*	*S*	*D*	Proportion of mice with tumors	Mean tumor diameter at 21 days ± SE
									mm
1	None			–				7/7	14.0 ± 0.9
	B10.A (1R)	*k*	*k*	*k*	*d*	*d*	*b*	6/6	16.3 ± 0.5
	B10.A (2R)	*k*	*k*	*k*	*d*	*d*	*b*	5/5	11.2 ± 1.3
	B10.A (4R)	*k*	*k*	*b*	*b*	*b*	*b*	6/6	14.25 ± 0.8
	B10.A (15R)	*k*	*k*	*k*	*d*	*d*	*b*	6/6	14.2 ± 0.7
2	None			–				15/15	14.0 ± 0.4
	AKR	*k*	*k*	*k*	*k*	*k*	*k*	10/10	13.8 ± 1.2
	ST	*k*	*k*	*k*	*k*	*k*	*k*	5/6	13.0 ± 2.6
	AL/N	*k*	*k*	*k*	*d*	*d*	*d*	9/9	13.4 ± 0.7

Discussion

The results presented in this paper clearly indicate that C3Hf mice do not normally express a tissue alloantigen readily detectable in other mice of known $H\text{-}2K^{k}$ haplotype. This antigen is, however, expressed on the C3Hf mouse-derived lung tumor 85 and, as reported elsewhere, is also expressed on several additional transplacentally induced lung tumors of C3Hf (15), C57BL/6, and DBA/2 mice (17). It is not expressed in normal tissues of C57BL/6, DBA/2, or other strains of mice that do not possess the $H\text{-}2K^{k}$ haplotype.

The induction of anti-lung tumor immunity in C3Hf mice with tissue from B10.BR (*kkkkkk*) and B10.A(4R) (*kkbbbb*) mice but not with tissue from either A.TL (*skkkkd*) or B10.AQR (*qkkddd*) mice indicates that the expression of the tumor cross-reactive normal tissue alloantigen is determined by a gene that is either within the *H-2K* region of the MHC or to the left of the *H-2K* region. Because there are relatively few commonly occurring genetic markers to the left of the MHC, the precise location of crossing over in this region for many of the recombinant strains used is unknown (18, 19). Very close linkage or identity of the genes coding for the lung tumor-associated alloantigen and the H-2K^{k} antigen is, however, supported by the detection of the tumor-associated antigen in H-2^{k} mice of diverse origins. Thus, although a common parental stock can be identified in the early derivation of strains C3H, CBA, and A mice, no clear relationship exists between the origin of these strains and either RF, St/b, or Ma/My strains (18). It is interesting that the normal H-2^{k}-associated alloantigen is readily detected in CBA (532) strain mice. Mice of this strain appear to have deviated from normal CBA mice in their expression of an *H-2K* region-coded antigen (20). The MHC haplotype of the strain has been designated $H\text{-}2^{ka}$ (21). Because the apparent alteration in the *H-2K* region of C3Hf mice is distinct from that of CBA (532) mice and because the H-2^{ka} strain is the only previously described mutation in the *H-2K* region of H-2^{k} mice, we propose that the *H-2* haplotype of the C3Hf strain be provisionally designated H-2^{kb}. As with several of the known mutations in the *H-2K* region of the MHC (22), we have been unable to detect serological differences between the H-2K-coded antigen on C3H and C3Hf mice. Thus spleen cells from both of these strains are comparably susceptible to lysis by antisera directed against the private specificity of the H-2K^{k}-coded antigen (unpublished observations). The known public specificities of the H-2K^{k} molecule are unlikely to be involved because mice of the *H-2*^{r} haplotype express the same *K* region-coded public H-2 specificities as H-2^{k} mice (18). Attempts to raise antisera specific for a *H-2K* region-coded molecule by cross immunizing C3H and C3Hf mice have to date not been successful. These serological studies need to be extended, however, before it can be concluded that no serologically detectable difference exists between the H-2K molecule of C3H and C3Hf mice. The more crucial question of whether the lung tumor-associated normal tissue alloantigen is an antigenic determinant on the serologically defined H-2K molecule or a cell surface component distinct from the serologically defined H-2K-coded molecule must await detailed structural studies of the serologically defined H-2K-coded antigen of C3H and C3Hf mice. Similarly, it is premature to conclude whether the expression of the alloantigen on the lung tumor 85 is the result of derepression of a genetic locus coding directly for the alloantigen or for an enzyme (e.g. a glycosyl transferase) or for some other entity (e.g. a type C viral antigen) capable of modifying the antigenicity of a preexisting cell surface component. Experimental resolution of these issues should provide important insights into the complexity of the genetic regulation of expression of MHC-coded alloantigens.

The studies reported in this and related papers reflect an attempt to test experimentally whether genetically determined tumor susceptibility can be correlated with the expression of specific alloantigens on normal tissues. It was reasoned that it was advantageous to the host that certain genetic loci coding for cell surface antigens be repressed in normal, but not in malignant, cells. Derepression of an antigen on malignant cells would enable the host to challenge immunologically the aberrant cell. Only in strains of mice that did not express the antigen in normal tissue could that antigen function as a target for immune surveillance (3, 14). The expression of a strain A-associated alloantigen on lung tumors induced in C3Hf mice was consistent with the hypothesis, because strain A mice are far more susceptible than C3Hf mice to the development of spontaneously occurring lung tumors (3, 9, 21). This interpretation cannot, however, be reconciled with the evidence obtained in the studies presented in this paper. Thus, B10.A strain mice and most strains of H-2^{k} mice have a low incidence of spontaneously occurring lung tumors (23 and unpublished observations). Furthermore, BALB/c mice, which are relatively susceptible to lung tumors (23), do not express the lung tumor 85-associated alloantigen in their normal tissue. Nevertheless, it is clear that transplancentally induced lung tumors may express foreign MHC-coded alloantigens which are highly immunogenic in syngeneic mice. Furthermore, although not necessarily MHC coded, the TATA on several methylcholanthrene-induced sarcomas have been identified by Invernizzi and Parmiani (24) as derepressed or altered alloantigens. Possibly a more meaningful consideration of the biological relevance of altered alloantigen expression to tumor susceptibility may be to relate genetic resistance to tumor development with the propensity to express new MHC-coded alloantigens. In preliminary studies we have observed that lung tissue derived from C3Hf mice 4 to 6 wk after ethyl nitrosourea administration was capable of immunizing C3Hf but not (C3Hf × A)F_1 hybrid mice, against the lung tumor 85. It will be of interest to pursue these studies to determine whether the lung tumor 85-associated antigen can be induced in other tissues of C3Hf mice after carcinogen administration, and to compare strain A and B10.A mice for any carcinogen-induced alteration of lung tissue expression of the *H-2K* region-coded antigen.

Another aspect of the studies reported in the paper relates to the now well-established finding that *H-2K* and *H-2D* region-coded antigens determine the susceptibility of virus-infected target cells to lysis by antiviral cytotoxic T lymphocytes. Thus *H-2K* and/or *H-2D* compatibility between cytotoxic T lymphocyte and virus-infected target cell is generally required for immune lysis to occur (6–10). Evidence has recently been reported which strongly favors the notion that cytotoxic T-lymphocyte recognition of MHC-coded antigens occurs independently from recognition of viral antigens (25). The phenomenon of MHC-restricted lysis of viral-infected target cells is relevant to a consideration of whether immune surveillance occurs against altered MHC-coded antigens of tumors. Presumably, if immune surveillance against tumors is achieved by recognition of altered or derepressed MHC-coded antigens, clones of lymphocytes bearing receptors for these new antigens would be selectively expanded in normal mice. Assuming lymphocytes possess two distinct sets of independently segregating receptors (25, 26), it would follow that in normal mice most lymphocytes bearing a receptor for any given antigen (e.g. viral antigen) would have their second receptor directed against an altered MHC-coded antigen. Optimal lysis would be expected to occur when the viral-infected target cells were recognized by lymphoid cells bearing one set of receptors for the viral antigen and the other set of receptors for the viral-induced altered MHC-coded antigen. Thus, the *H-2K* and/or *H-2D* compatibility requirement for immune lysis of viral-infected target cells could support the notion of anti-tumor immune surveillance, providing the same altered MHC-coded antigens specify anti-tumor and anti-viral immunity. This possibility can be tested by determining whether viral infection of C3Hf-derived cell lines can cause the expression of the C3H-associated alloantigen. In this regard it is interesting that Garrido and his colleagues (27, 28) have demonstrated foreign H-2-like specificities on tumor cells after vaccinia virus infection. More extensive studies are clearly needed to define fully the interrelationship between anti-viral and anti-tumor immunity. The C3Hf mouse lung tumor model system described in the paper should be useful in this endeavor and should encourage more direct studies on the regulation of MHC-coded antigen expression in other tumor systems.

Summary

Transplacental induction of lung tumors in C3HfeB/HeN (C3Hf) strain mice can be readily achieved with the carcinogen 1-ethyl-1-nitrosourea. Several of these tumors express, as a tumor-associated transplantation antigen (TATA), a

normal tissue alloantigen present in strain A and C3H/HeN (C3H) mice. In the present study it was shown that the tumor-associated alloantigen on the C3Hf-derived lung tumor 85 was present in all mice of *H-2*^{a} and *H-2*^{k} haplotypes tested and in CBA (532) strain mice (*H-2*^{ka} haplotype). Studies using congenic-resistant and recombinant strains of mice indicated that the genetic locus controlling the expression of this antigen was either within or to the left of the *H-2K* region of the major histocompatibility complex (MHC). Thus the antigen was expressed in B10.A (4R) mice (*kkbbbb* MHC haplotype) but not in B10 (*bbbbbb*) or B10.AQR mice (*qkkddd*). The antigen was expressed in all tissues tested of C3H and A strain mice. It was not detected on any tissue tested including embryo tissue of C3Hf mice or mice of MHC haplotype other than *H-2*^{k} or *H-2*^{a}. Because C3Hf strain mice were originally derived from C3H strain mice (H-2^{k}), the MHC haplotype of C3Hf mice has been provisionally designated *H-2*^{kb}. The finding of a tumor-associated change in the expression of a *H-2K* region-coded antigen is consistent with the concept that MHC-coded antigens may act as targets for immunological surveillance of tumors.

We wish to thank Dr. Jerry Rice for his support of these studies, Rick Wallace and Mike Falco for their technical assistance, and Roseanna Bell for her secretarial assistance.

Received for publication 2 December 1977.

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THE RELATIONSHIP BETWEEN SURFACE IMMUNOGLOBULIN ISOTYPE AND IMMUNE FUNCTION OF MURINE B LYMPHOCYTES

III. Expression of a Single Predominant Isotype on Primed and Unprimed B Cells*

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There are now several lines of evidence which indicate that B cells bearing different surface immunoglobulin isotypes are functionally distinct subsets of cells (1–10). Thus, negative (5) and positive (3, 4, 8, 9) selection studies as well as studies utilizing anti-Ig[1] reagents to block immune responses in vitro (1, 2, 6, 7, 10) suggest that cells bearing IgM or IgD give rise to an IgM antibody response followed by an IgG response. In contrast, cells bearing IgG give rise only to an IgG response (3–6, 8, 9) and suppress the IgM response of the cells bearing IgM and IgD (8, 9).

Because the majority of splenic B cells in the mouse bear more than one surface Ig isotype (11–14), a further delineation of B-cell subsets requires the use of additional techniques to determine the function of cells bearing one as opposed to multiple isotypes. For example, it has been hypothesized previously (15) that cells bearing both IgM and IgD give rise to an IgM response, and that cells bearing only IgD give rise only to an IgG response.

The goal of the present study was to determine whether B cells in the mouse spleen, which restore the adoptive antibody response to bovine serum albumin (BSA), contain subpopulations that express a single predominant Ig isotype. The experimental results reported here show that subpopulations of primed and unprimed B cells bearing predominantly IgM, IgD, or IgG (μp, δp, or γp cells) are found in the spleen but in lower quantities than those subpopulations that

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* Supported by National Institutes of Health grants AI 10293, 11851, and 12789.

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; FACS, fluorescence-activated cell sorter; Ig, immunoglobulin; FCS, fetal calf serum; 2-ME, 2-mercaptoethanol; MEM, Eagle's minimum essential medium; PBS, phosphate-buffered saline; RAMIg, rabbit anti-mouse Ig serum; RAδ, rabbit anti-mouse δ-chain antiserum; RAγ, rabbit anti-mouse γ-chain antiserum; RAμ, rabbit anti-mouse μ-chain antiserum, μp, γp, δp cells, cells bearing predominantly surface IgM, IgG, or IgD, respectively.

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express a combination of isotypes. The IgD-predominant cells differ from cells bearing combinations of IgM and IgD ($\mu^+\delta^+$) in that the former cells are found only in primed donors, and give rise only to the adoptive IgG antibody response. The latter cells are found in both unprimed and primed donors, and give rise to the adoptive IgM and IgG responses. IgG-predominant cells, also present in unprimed and primed animals, give rise to an adoptive IgG response, and (unlike the δp cell) suppress the adoptive IgM response of IgM and IgD bearing cells. The relationships of the several B-cell subpopulations are discussed in the context of antigen-dependent and independent B-lymphocyte maturation.

Materials and Methods

Experimental Design. To prepare cells bearing a single Ig isotype, spleen cells were stained with a combination of antisera against two of the three major surface isotypes (e.g., anti-μ and anti-δ), and the positive cells were depleted by sorting on the fluorescence-activated cell sorter (FACS). The remaining B cells that express the surface isotype not stained for were used as the source of isotype-predominant (e.g., γp) cells.

In cell transfer studies of the adoptive secondary response, spleen cells from (BALB/c × C57BL/Ka)F_1 mice immunized to DNP-BSA (Calbiochem, San Diego, Calif.) 8–12 wk earlier were sorted on the FACS and injected into syngeneic irradiated (650 rads) recipients. An excess of nylon wool-purified cells from donors immunized to BSA 8–12 wk earlier (helper T cells) were given to recipients, and the adoptive hosts were challenged within 24 h with DNP-BSA in saline. Serum antibodies to both BSA and DNP were measured for 2 wk thereafter. Control recipients received either 2×10^6 or 0.4×10^6 unfractionated DNP-BSA-primed spleen cells, and experimental recipients received a dose of bright (positively stained) or dull (negatively stained) cells from the FACS contained within 2×10^6 or 0.4×10^6 unfractionated cells. These cell doses were previously determined to be on the linear portion of a dose-response curve constructed by transferring graded numbers of anti-Thy 1.2-treated DNP-BSA-primed spleen cells to irradiated recipients given an excess of BSA-primed T cells (8). The anti-DNP and anti-BSA responses are both dependent on the presence of carrier-primed T cells and primed B cells (8).

In cell transfer studies of the adoptive primary response, spleen cells from unprimed mice were stained with various combinations of rabbit anti-mouse Ig isotype antisera, sorted on the FACS, and transferred to syngeneic irradiated (750 rads) recipients. Adoptive hosts were given an excess of nylon wool-purified T cells (5×10^6 cells) from unprimed donors, and challenged subcutaneously within 24 h with DNP-BSA in complete Freund's adjuvant. Serum antibodies to both BSA and DNP were measured for 4 wk thereafter. Control recipients received either 4 or 0.8×10^6 unfractionated cells, and experimental recipients received a dose of bright or dull cells contained within either 4 or 0.8×10^6 unfractionated cells. Our previous studies (9) show that the adoptive primary responses are completely dependent upon the transfer of the nylon wool T cells, and that the spleen cell doses chosen are on the linear portion of a dose-response curve constructed by transferring graded numbers of unprimed spleen cells treated in vitro with anti-Thy 1.2 antisera to irradiated recipients given an excess of T cells. Results of the adoptive primary and secondary anti-BSA response from representative experiments are reported here. Results of the anti-DNP response will be the subject of a separate communication.

Antisera

Rabbit Anti-Mouse-μ (RAμ; 16). This serum was prepared against μ-chains isolated from MOPC-104E and was monospecific for both serum and cell surface IgM. The IgG fraction of this serum was purified on DEAE-Sephadex A50 (Pharmacia Fine Chemicals, Piscataway, N. J.) as described previously (8), and used for staining of cell surface IgM as outlined below.

Rabbit Anti-Mouse-δ (RAδ). This serum was made against a purified fraction of spleen cell surface Ig by a modification of the technique of Abney et al. (12) as described by Zan-Bar et al. (8). Details of the preparation of the membrane extract, immunizations, and absorption procedures, and assays for specificity have been published elsewhere (8). The batch of anti-δ used in these studies was extensively absorbed with thymocytes before use. In brief, the specificity of the antiserum used in the present report was documented as follows: (*a*) precipitation of only the "δ" and "L" chain peaks of lysates of radioiodinated splenocytes as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (8); (*b*) inability to bind radioiodinated IgM, IgG, and IgA myeloma proteins (8); (*c*) binding to the surface of spleen cells that bear IgM with redistribution (capping) of surface determinants independently of surface IgM (8); (*d*) binding to spleen cell surface determinants that co-cap with surface Ig identified with rabbit anti-mouse Ig antiserum (8); (*e*) failure to stain thymocytes (8); (*f*) failure to stain spleen cells previously stripped with either anti-Ig (M. Knapp, F. Assisi, and S. Strober, unpublished observations) or allotypic (17) anti-δ (F. Ligler, E. S. Vitetta, and J. Goding, unpublished observations).

Rabbit Anti-Mouse γ(RAγ; 5). This serum was made against γ-chains (prepared from reduced and alkylated serum IgG) and was monospecific for IgG. The $F(ab')_2$ fragment of the IgG fraction of the serum was prepared as described previously (8), and used in all cell surface staining procedures in order to minimize binding via the Fc receptor.

Rabbit Anti-Mouse Ig (RAMIg; 16). This antiserum contains antibodies against μ-, γ-, α-, κ-, and λ-chains and is a pool of rabbit sera prepared against MOPC-104E (μ,λ), TEPC-15 (α,κ), and serum IgG.

Immunofluorescent Staining for Surface Ig. Spleen cells were stained for surface Ig with rabbit anti-mouse Ig isotype antisera, and counter-stained with the IgG-fraction of fluoresceinated goat anti-rabbit-IgG antiserum (Meloy Laboratories Inc., Springfield, Va.). The latter antiserum was thoroughly absorbed with thymocytes before use. Spleen cells were suspended in tissue culture medium 199 with 5% fetal calf serum (FCS) at a concentration of 20×10^6 cells/ml during all incubations. In some experiments cells were stained for three surface isotypes (IgM, IgD, and IgG) by either sequential incubations with RAμ, RAδ, and RAγ at 4°C for 30 min each, or by a single incubation with a combination of all three antisera. Similar procedures were used to stain cells for surface isotypes (IgM and IgD; IgM and IgG; or IgD and IgG) by incubating cells with RAμ and RAδ, RAμ and RAγ, or RAδ and RAγ, respectively. After incubation with rabbit antisera, the cell suspension was layered on FCS and centrifuged for 10 min at 150 *g*. The cell pellet was resuspended in culture medium with fluoresceinated goat anti-rabbit IgG antiserum for 30 min at 4°C, and washed before sorting or analysis.

All rabbit antisera were used for staining at a dilution determined to give the optimum percentage of positive cells over a 40-fold range of concentrations tested (4-0.1 mg/ml). RAγ was used at 1 mg/ml, RAδ at 2 mg/ml, and RAμ at 0.5 mg/ml. The optimum concentration of the goat antiserum was similarly chosen.

Sorting and Analysis of Cells with FACS. Stained spleen cells were analyzed for the percentage of bright fluorescent cells, and sorted on the same basis using the FACS (18). Thresholds were set so that bright cells corresponded to positive cells as judged by fluorescence microscopy. For purposes of sorting, the next brightest 10% of cells were discarded, and the remaining cells constituted the dull cell fraction. Details of the sorting procedure have been described elsewhere (19). Approximately 40–50% of spleen cells were recovered in the combined bright and dull cell fractions. Bright cells were contaminated with up to 5% of dull cells, and dull cells were contaminated with up to 3% of bright cells as judged by repeat analysis of sorted cells. Approximately 5×10^7 cells were sorted in each experiment at a rate varying between 3 and 5×10^3 cells/s.

In the cell transfer experiments, spleen cells were stained in culture medium that contained either a single anti-isotype antiserum, a combination of two anti-isotype antisera, or a combination of all three anti-isotype antisera (anti-μ + anti-γ + anti-δ). Cells were counterstained with a fluorescein-conjugated goat anti-rabbit Ig antiserum and sorted on the FACS. Experiments using all three antisera (Figs. 1 and 7) showed that the dull cells (IgM^-, IgD^-, IgG^-) were unable to restore a detectable primary or secondary response to BSA, and the bright cells (IgM^+ + IgD^+ + IgG^+) restored a response similar to that of unfractionated cells. It was, therefore, concluded that any restorative activity found in the dull cells after staining with a combination of two antisera is due to residual B cells which bear predominantly the isotype not stained for. For example, B cells contained within the dull cell fraction after staining with anti-μ and anti-δ antisera are those cells that express predominantly IgG (γp). The isotype-predominant cells may bear small quantities of other isotypes that are below thresholds of detection by immunofluorescence microscopy. It is for this reason that the term "isotype predominant" is used.

The adoptive responses of these isotype-predominant cells (contained in the dull cell fraction) were compared to those bright cells obtained after staining for the same isotype with a single antiserum. The latter cells (isotype-positive cells) contain both the isotype-predominant cells, and cells that express that isotype in combination with others.

Animals. (BALB/c × C57BL/Ka)F_1 mice obtained from the colony of Dr. R. Kallman, Department of Radiology, Stanford University School of Medicine, Stanford, Calif., were used in all experiments. Only female mice, 2 to 3 mo old, were selected for investigation.

Irradiation of Mice. Mice were placed in Lucite containers and given either 650 rads (secondary response) or 750 rads (primary response) whole body X-irradiation from a single 250 kV (15 Å) source. The dose rate was 54 rads/min (0.25 mm Cu plus 0.55 mm Al filtration) with a 52-cm source axis distance. Cells were transferred to adoptive hosts 4–6 h after irradiation.

Immunization Procedures. Donors of carrier-primed cells were immunized with a subcutaneous injection (0.2 ml) of an emulsion of equal volumes of BSA (Calbiochem) in saline and complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). Each animal received a total dose of 0.5 mg protein. Donors of hapten (DNP)-primed cells were immunized with DNP_{18}-BSA in complete Freund's adjuvant as described above. BSA was dinitrophenylated with DNP-benzene sulfonate as reported previously (20).

In studies of the adoptive secondary response, irradiated recipients were challenged intraperitoneally with 200 μg DNP-BSA in saline 1 day after cell transfer. Recipients used in the adoptive primary response were challenged subcutaneously with DNP-BSA in Freund's adjuvant as noted above.

Preparation of Cell Suspensions. Cell suspensions were prepared by mincing lymph nodes and spleens with scissors, and gently pressing the fragments through a nylon mesh. Cells were harvested by centrifugation at 150 *g*, and resuspended in Eagle's Minimum Essential Medium without sodium bicarbonate (MEM, Grand Island Biological Co., Grand Island, N. Y.) before intravenous injection into the lateral tail vein of irradiated recipients.

Passage of Lymph Node and Spleen Cells Over Nylon Wool Columns. T lymphocytes from the spleen and lymph nodes were purified by passage over a nylon wool column as described by Julius et al. (21). Cell suspensions were applied to LP-1 Leuko-Pak filters (Fenwall Electronics, Framingham, Mass.) equilibrated with 5% FCS in MEM and maintained at 37°C. The percentage of T cells in the effluent was >95% as judged by killing with anti-Thy 1.2 antiserum. The percentage of Ig-bearing cells (B cells) was <5% as judged by immunofluorescent staining with RAMIg.

Collection of Serum Samples. Blood samples were collected from the retro-orbital vein. Serum from each sample was separated by centrifugation and stored at −20°C.

Antibody Titrations. Antibodies to BSA were measured by a tanned red blood cell hemagglutination procedure (22). Serial twofold dilutions of serum were made in microtiter plates (Cooke Laboratory Products Div., Dynatech Laboratories Inc., Alexandria, Va.) in 1% normal rabbit serum. The total and 2-mercaptoethanol (2-ME)-resistant antibody titer or each serum sample was measured simultaneously. The latter titer was determined by incubation of serum with an equal volume of 0.1 M 2-ME in saline for 30 min at room temperature before serial dilution. Antibody determinations are expressed as the $\log_2$ of the reciprocal of the final dilution of serum showing a smooth carpet of agglutinated cells. The titer of 2-ME-sensitive antibody = $\log_2$ titer total antibody − $\log_2$ titer 2-ME-resistant antibody.

Results

Analysis of the Percentage of Spleen Cells Stained with Anti-Isotype Antisera Using the FACS. Spleen cells were stained with combinations of anti-isotype antisera, and sorted on the FACS to determine whether cells bearing a single predominant Ig isotype could be identified. Results of representative experiments are shown in Table I. In experiment 1 (Table I), cells were stained with RAμ, RAδ, and RAγ and sorted on the FACS to deplete the ($\mu^+ + \delta^+ + \gamma^+$) cells. The residual dull cells were restained with all four antisera (RAMIg, RAμ, RAδ, and RAγ). The percentage of Ig^+, μ^+, δ^+, or γ^+ cells was <2% above the background level. The results of experiment 2 (Table I) show that depletion

TABLE I

Percentage of Spleen Cells Staining Positively for Surface Ig Isotype

Exp	Cells depleted by sorting on FACS	Positive cells before depletion (unfractionated cells)	Positive cells after depletion (dull cells)[*]	Positive cells corrected for background staining
		%	%	%
1	$\mu^+ + \delta^+ + \gamma^+$	Total Ig - 58.3	2.53	0.96
		μ^+ - 32.5	2.55	0.98
		δ^+ - 50.5	3.40	1.83
		γ^+ - 8.2	3.10	1.53
		BKG[‡] - 0.5	1.57	–
2	$\delta^+ + \gamma^+$	Total Ig - 48.2	7.1	5.4
		μ^+ - 32.6	6.7	5.0
		δ^+ - 26.1	2.6	0.9
		γ^+ - 11.2	2.0	0.3
		BKG[‡] - 0.5	1.7	–
3	$\mu^+ + \gamma^+$	Total Ig - 63	11.9	10.2
		μ^+ - 51	2.3	0.6
		δ^+ - 28	8.6	6.9
		γ^+ - 9.5	1.9	0.2
		BKG[‡] - 1.5	1.7	–
4	$\mu^+ + \delta^+$	Total Ig - 60.9	4.1	2.4
		μ^+ - 31.0	1.2	0
		δ^+ - 28.2	1.3	0
		γ^+ - 14.9	2.8	1.1
		BKG[‡] - 1.2	1.7	–

[*] Dull cells accounted for 42, 60, 43, and 40% of unfractionated cells in experiments 1-4, respectively.

[‡] Background staining with second stage alone.

of ($\delta^+ + \gamma^+$) cells leaves a residual population of 6.7% μ^+ cells (background-1.7%) which accounts for almost all of the Ig-positive cells (7.1%). The percentage of μ^+ cells is disproportionately higher than that of the δ^+ (2.6%) and γ^+ (2.0%) cells. This indicates that there is a subpopulation of spleen cells that are μ^+, but that do not bear quantities of surface IgD or IgG above the threshold set on the FACS and corresponding to detection by the naked eye. We have defined these cells as μ-predominant (μp) cells.

Experiment 3 (Table I) shows that depletion of ($\mu^+ + \gamma^+$) cells leaves a residual population that contains a disproportionately high percentage (8.6%) of δ^+ cells (background-1.7%) as compared to μ^+ (2.3%) or γ^+ (1.9%) cells. The percentage of μ^+ and γ^+ cells was <1% above background. Thus, a small population of δp as well as μp cells is found in the adult spleen.

The results of experiment 4 suggest that there is a subpopulation of splenic γp cells because the percentage of γ^+ cells (2.8%) after depletion of ($\mu^+ + \delta^+$) cells is above that of the μ^+ (1.2%), δ^+ cells (1.3%), and background (1.7%). However, the size of this subpopulation is variable (1-4%) and falls within the limits of error (1-2%) of our analysis of stained cells in most experiments.

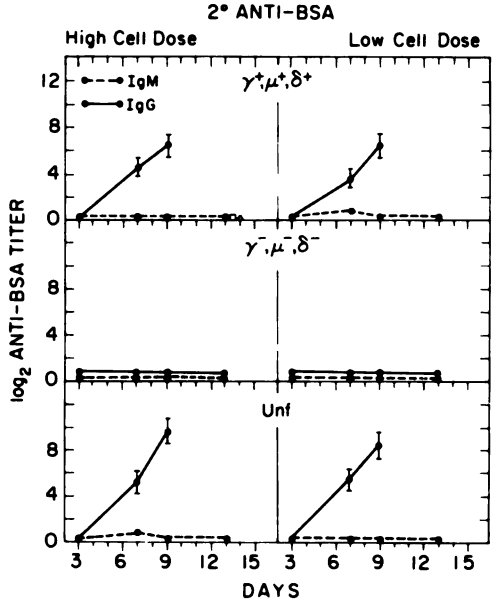

FIG. 1. Adoptive secondary anti-BSA response restored by (μ^+ + δ^+ + γ^+) spleen cells. Cells were stained with a combination of RAμ, RAδ, and RAγ, and separated on the FACS. Irradiated mice received a dose of bright (μ^+ + δ^+ + γ^+) or dull (μ^-, δ^-, γ^-) cells contained within 2 or 0.4 × 10^6 unfractionated cells. All recipients were given 5 × 10^6 BSA-primed T cells. Controls received 2 or 0.4 × 10^6 unfractionated (unf) spleen cells plus T cells or T cells alone. △, 0.02 × 10^6 (μ^+ + δ^+ + γ^+) cells (dose of bright cells contaminating dulls); □, T cells alone. Each point represents the mean response of a group of four to eight mice, and brackets show the standard error. Log_2 titer 2-ME-sensitive antibody (IgM) = $\log_2$ titer total antibody − $\log_2$ titer 2-ME-resistant antibody (IgG).

Nevertheless, functional studies discussed below show that γp cells can be readily detected by an independent assay system.

The variability of the percentage of μ^+ and δ^+ cells in the adult spleen before fractionation (Table I) has been observed previously (8, 9). This variation occurs between different groups of mice, but repeat staining and analysis of the spleen cells from a single group performed on the same day give percentages that are within the limits of error noted above.

The actual percentage of isotype-predominant cells in the whole spleen is lower than that shown in the dull cell fractions in Table I, because the dull cells represent only a portion of the whole spleen cell population. Accurate calculation of the percentage of isotype-predominant cells in the spleen is difficult, because there is a gap between the thresholds of the bright and dull cells due to our efforts to minimize contamination of the sorted cells. However, if one assumes that the dull cell population is homogeneous and contains all cells not stained positively, then the maximum net percentage of δp, μp, and γp cells in the whole spleen is 3–4, 2–3, and 0.5–1%, respectively. As approximately 55% of splenic lymphocytes are B cells (8, 9), 11–16% of these B cells bear only one surface Ig isotype.

Secondary Anti-BSA Response Restored by Splenic B Cells. Fig. 1 shows the anti-BSA response restored by spleen cells stained for all three surface isotypes

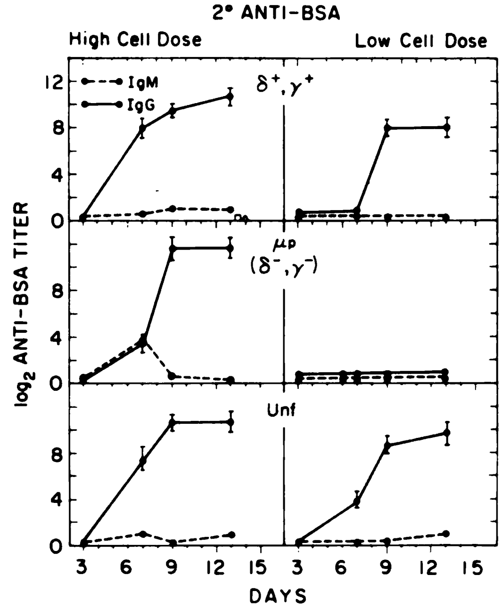

FIG. 2. Adoptive secondary anti-BSA response restored by ($\delta^+ + \gamma^+$) spleen cells. Cells were stained with a combination of RAδ and RAγ, and separated on the FACS. Irradiated mice received a dose of bright ($\delta^+ + \gamma^+$) or dull (δ^-, γ^-) cells contained in 2 or 0.4 × 10^6 unfractionated cells. All recipients were given 5 × 10^6 BSA-primed T cells. Controls received 2 or 0.4 × 10^6 unfractionated cells plus T cells or T cells alone. △, 0.04 × 10^6 ($\delta^+ + \gamma^+$) cells (dose of bright cells contaminating dulls); □, T cells alone. Each point represents the mean response of a group of four to eight mice, and brackets show the standard error.

(μ, γ, δ) and sorted on the FACS. The kinetics and magnitude of the response produced by the ($\mu^+ + \delta^+ + \gamma^+$) cells were similar to those of the unfractionated cells. Anti-BSA antibodies in both cases were all IgG (2-ME resistant) at both days 7 and 9. Spleen cells depleted of all three isotypes produced no detectable anti-BSA response by day 9. These results indicate that staining cells with a combination of all three anti-isotype antisera depletes all functional B cells as efficiently as staining with a polyvalent anti-Ig (8).

Secondary Anti-BSA Response Restored by μp Cells. Fig. 2 shows the anti-BSA response restored by spleen cells stained for both surface "δ" and "γ," and sorted on the FACS. The ($\delta^+ + \gamma^+$) cells restored an IgG response that was similar to that of the unfractionated cells. On the other hand, the (δ^-, γ^-) cells (μp cells) restored both an IgM and IgG response. The IgM antibody (2-ME sensitive) was predominant on day 7 and accounted for approximately 90% of the measured antibody (Table II). All antibody was IgG by day 9. Although a substantial response was produced by both ($\delta^+ + \gamma^+$) and unfractionated cells at the low cell dose (0.4 × 10^6 cells), no response was observed with a similar dose of (δ^-, γ^-) cells. These results indicate that the μp cells given in sufficient numbers can generate adoptive IgM and IgG responses.

Secondary Anti-BSA Response Restored by δp Cells. Fig. 3 shows the anti-BSA response restored by ($\mu^+ + \gamma^+$) and (μ^-, γ^-) (δp) spleen cells. Antibodies produced by the ($\mu^+ + \gamma^+$) and unfractionated cells were all IgG, and showed

TABLE II
Relative Quantity of 2-ME-Sensitive and 2-ME-Resistant Anti-BSA Antibody on Day 7 of the Adoptive Secondary Response

Surface Ig isotype of donor cells*	Mean total antibody response ($\log_2$ titer)‡	Percentage of 2-ME-resistant (IgG) antibody
		%
μp	6.6	10
δp	6.5	95
γp	9.3	100
μ^+	9.3	2
δ^+	6.9	8
γ^+	8.7	100
$(\mu^+ + \delta^+)$	4.0	13
$(\mu^+ + \gamma^+)$	4.9	100
$(\mu^+ + \delta^+ + \gamma^+)$	4.6	100
$(\delta^+ + \gamma^+)$	8.0	100

* μ^+, δ^+, γ^+ denotes cells staining positively for given isotype. μp, δp, γp denotes cells bearing predominantly single isotype.

‡ Adoptive antibody response restored by high dose of cells.

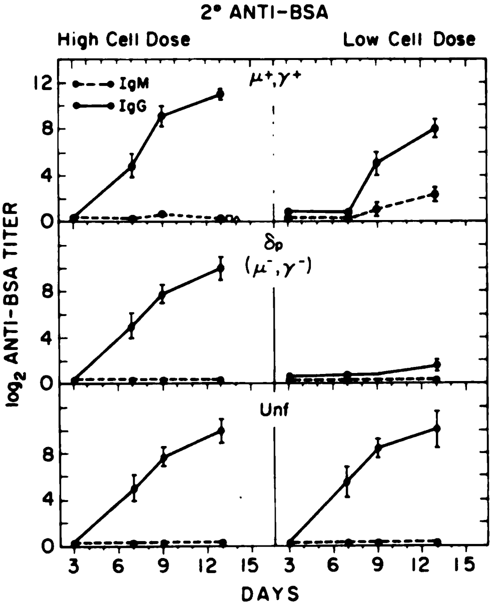

FIG. 3. Adoptive secondary anti-BSA response restored by $(\mu^+ + \gamma^+)$ spleen cells. Cells were stained with a combination of RAμ and RAγ, and separated on the FACS. Irradiated mice received a dose of bright $(\mu^+ + \gamma^+)$ or dull (μ^-, γ^-) cells contained in 2 or 0.4 × 10^6 unfractionated cells. All recipients were given 5 × 10^6 BSA-primed T cells. Controls received 2 or 0.4 × 10^6 unfractionated cells plus T cells or T cells alone. △, 0.04 × 10^6 $(\mu^+ + \gamma^+)$ cells (dose of bright cells contaminating dulls); □, T cells alone. Each point represents the mean response of a group of four to eight mice, and brackets show the standard error.

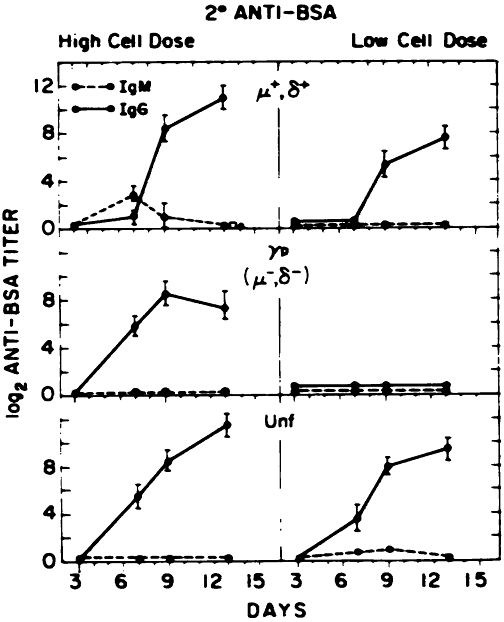

FIG. 4. Adoptive secondary anti-BSA response restored by $(\mu^+ + \delta^+)$ spleen cells. Irradiated mice received a dose of bright $(\mu^+ + \delta^+)$ or dull (μ^-, δ^-) cells contained within 2 or 0.4 × 10^6 unfractionated cells. All recipients were given 5 × 10^6 BSA-primed T cells. Δ, 0.03 × 10^6 $(\mu^+ + \delta^+)$ cells (dose of bright cells contaminating dulls); □, T cells alone. Each point represents the mean of a group of four to eight mice, and brackets show the standard error.

similar titers on days 7 and 9 for both cell doses. Although the response restored by (μ^-, δ^-) (δp) cells was similar to that of the $(\mu^+ + \gamma^+)$ cells at the high cell dose, δp cells made no response at the low cell dose. These results indicate that δp cells generate only an IgG response.

Secondary Anti-BSA Response Restored by γp Cells. The anti-BSA response restored by $(\mu^+ + \delta^+)$ cells (Fig. 4) differed from that of the $(\mu^+ + \gamma^+)$, $(\gamma^+ + \delta^+)$, and $(\mu^+ + \delta^+ + \gamma^+)$ cells in that the former cells produced a predominantly IgM response at day 7 (Table II), and the latter cells produced only IgG antibody. The (μ^-, δ^-) cells (γp) produced only IgG antibody with kinetics similar to that of unfractionated cells at the high cell dose. However, the γp cells (as well as δp and μp cells) produced no detectable response at the low cell dose. These results indicate that γp cells generate an IgG response, and suppress the IgM response of μ^+ and δ^+ cells.

Comparison of the Adoptive Secondary Anti-BSA Response Restored by Isotype-Positive and Isotype-Predominant Cells. Figs. 5 and 6 compare the adoptive IgM and IgG anti-BSA responses restorod by μ^+, δ^+, and γ^+ cells with those restored by μp, δp, and γp cells, respectively, at high and low cell doses. The isotype-positive cells $(\mu^+, \delta^+, \gamma^+)$ were purified by staining with a single anti-isotype antiserum and sorting on the FACS. Fig. 5 shows that the kinetics and magnitude of the responses produced by μp and μ^+, and γp and γ^+ cells, respectively, are similar. In contrast, the response produced by the δ^+ cells

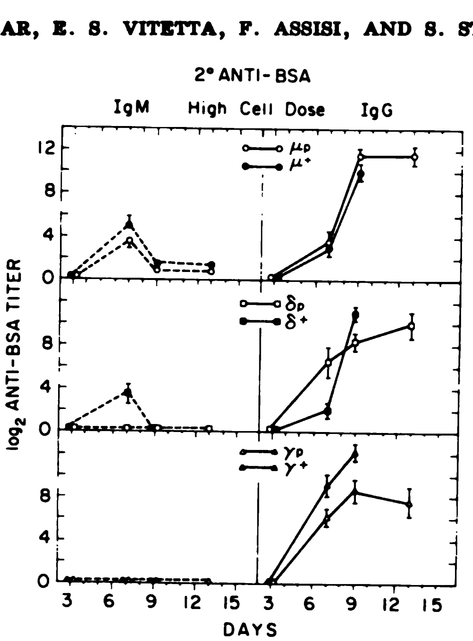

FIG. 5. Comparison of the adoptive secondary anti-BSA responses restored by isotype-positive and isotype-predominant cells (high cell dose). Isotype-positive cells are in the bright cell fraction obtained after staining with a single anti-isotype antiserum. Isotype-predominant cells are in the dull cell fraction obtained after staining with two anti-isotype antisera. Irradiated recipients received a dose of μ^+, δ^+, γ^+, μp, δp, or γp cells contained within 2 × 10^6 unfractionated cells. All recipients were given 5 × 10^6 BSA-primed T cells. Each point represents the mean of a group of four to eight mice, and brackets show the standard error.

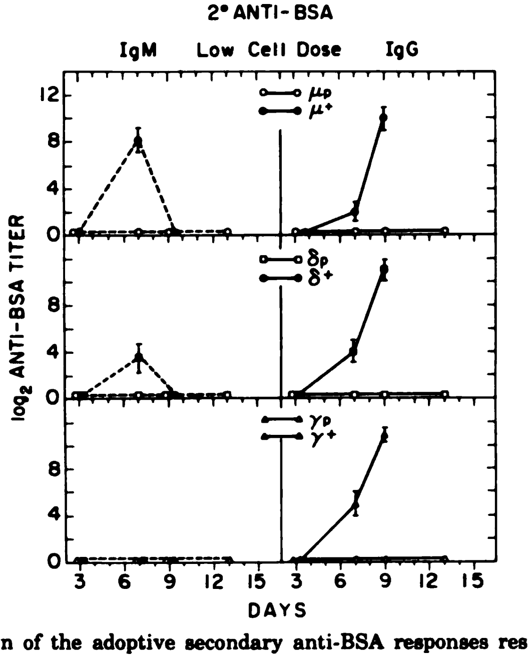

FIG. 6. Comparison of the adoptive secondary anti-BSA responses restored by isotype-positive and isotype-predominant cells (low cell dose). Irradiated recipients received a dose of μ^+, δ^+, γ^+, μp, δp, or γp cells contained within 0.4 × 10^6 unfractionated cells. All recipients were given 5 × 10^6 BSA-primed T cells. Each point represents the mean of a group of four to eight mice, and brackets show the standard error.

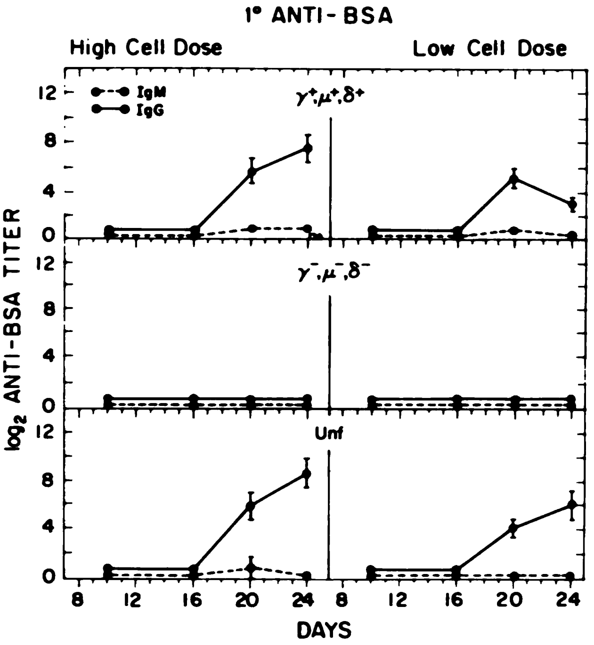

FIG. 7. Adoptive primary anti-BSA response restored by ($\mu^+ + \delta^+ + \gamma^+$) spleen cells. Cells were stained with a combination of RAμ, RAδ, RAγ and separated on the FACS. Irradiated mice received a dose of bright ($\mu^+ + \delta^+ + \gamma^+$) or dull ($\mu^-$, δ^-, γ^-) cells contained within 4 or 0.8×10^6 unfractionated cells. All recipients were given 5×10^6 unprimed T cells. Controls received 4 or 0.8×10^6 unfractionated (unf) spleen cells plus T cells or T cells alone. △, 0.08×10^6 ($\mu^+ + \delta^+ + \gamma^+$) cells (dose of bright cells contaminating dulls); □, T cells alone. Each point represents the mean response of a group of four to eight mice, and brackets show the standard error. Log_2 titer 2-ME-sensitive antibody (IgM) = $\log_2$ titer total antibody − $\log_2$ titer 2-ME-resistant antibody (IgG).

differed from that of the δp cells in that the former is predominantly IgM at day 7 (Table II) and the latter is all IgG. In addition, the 7-day IgG response of the δp cells is considerably greater than that of the δ^+ population. This suggests that subpopulations within the set of δ^+ cells may modulate the immune responses of one another.

Fig. 6 shows that the isotype-predominant cells were unable to restore the anti-BSA response at the low cell dose, but the same dose of isotype-positive cells restored a considerable response. A comparison of Figs. 5 and 6 shows that about five times as many isotype-predominant cells are required to give a response similar to that restored by a given number of isotype-positive cells. This indicates that the large majority of memory cells that contribute to the adoptive secondary anti-BSA response bear more than one surface Ig isotype.

Primary Anti-BSA Response Restored by Splenic B Cells. Fig. 7 shows the adoptive primary anti-BSA response restored by spleen cells stained for all three surface isotypes, and sorted on the FACS. The kinetics and magnitude of the response restored by the ($\mu^+ + \delta^+ + \gamma^+$) cells were similar to that of equivalent doses of unfractionated cells. The response was mainly IgG at days 20 and 25. The (μ^-, δ^-, γ^-) cells produced no detectable response at the high and

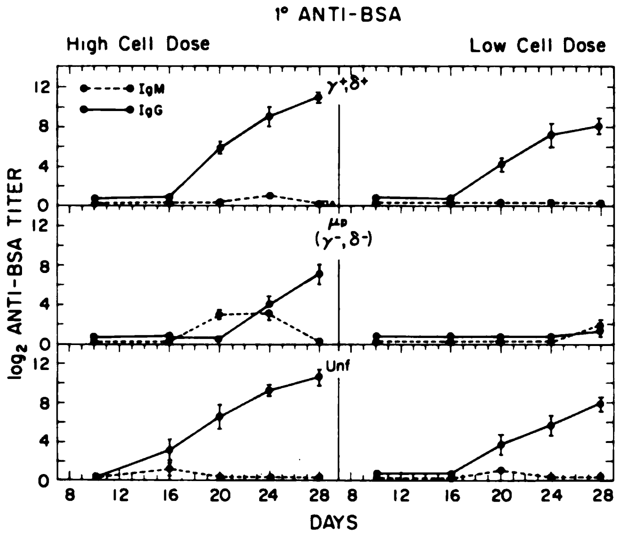

FIG. 8. Adoptive primary anti-BSA response restored by (γ^+ + δ^+) spleen cells. Cells were stained with a combination of RAδ and RAγ, and separated on the FACS. Irradiated mice received a dose of bright (γ^+ + δ^+) or dull (γ^-, δ^-) cells contained in 4 or 0.8 × 10^6 unfractionated cells. All recipients were given 5 × 10^6 unprimed T cells. Controls received 4 or 0.8 × 10^6 unfractionated cells plus T cells or T cells alone. △, 0.04 × 10^6 (γ^+ + δ^+) cells (dose of bright cells contaminating dulls); □, T cells alone. Each point represents the mean response of a group of four to eight mice, and brackets show the standard error.

low cell doses. These results indicate that staining cells with a combination of anti-isotype antisera depletes all functional B cells as efficiently as staining with polyvalent anti-Ig (9).

Primary Anti-BSA Response Restored by μp Cells. Fig. 8 shows that the (δ^+ + γ^+) spleen cells restored a vigorous anti-BSA response at both cell doses similar to that restored by the unfractionated cells. Almost all antibody at day 20 was IgG. On the other hand, the ($\delta^-\gamma^-$) cells (μp) restored a vigorous response only at the high cell dose, and most of the antibody was IgM at days 20 and 24 (see Table III). However, all antibody was IgG at day 29. Thus, the μp cells, although present in small numbers, can give rise to both IgM and IgG responses.

Primary Anti-BSA Response Restored by γp Cells. The adoptive primary response produced by (μ^+ + δ^+) cells was mainly IgM at day 20 and switched to IgG by day 25 at the high cell dose (Fig. 9, Table III). The γp cells restored a detectable response only at the high cell dose. The latter response was all IgG (Fig. 9). Thus, γp cells are found in the unprimed animal and can give rise to an IgG response.

Primary Anti-BSA Response Restored by δp Spleen Cells. Fig. 10 shows that (μ^+ + γ^+) cells restored only an IgG response similar to that of unfractionated cells at days 20 and 25 at both cell doses. It is of interest that the former response was delayed about 4 days as compared with the latter. The δp cells failed to make a detectable response at both the high and low cell doses, and, thereby differed in their restorative activity from the μp and γp cells.

TABLE III

Relative Quantity of 2-ME-Sensitive and 2-ME-Resistant Antibody to BSA on Day 20 of the Adoptive Primary Response

Ig isotype on donor cells	Mean total antibody response (log$_2$ titer)*	Percentage of 2-ME-resistant (IgG) antibody
		%
μp	3.0	0
δp	–	–
γp	2.5	100
μ^+	5.9	13
δ^+	3.9	13
γ^+	5.4	100
(μ^+ + δ^+)	7.3	8
(μ^+ + γ^+)	5.4	100
(δ^+ + γ^+)	6.0	100
(μ^+ + δ^+ + γ^+)	6.0	70

* Adoptive antibody response restored by high dose of cells.

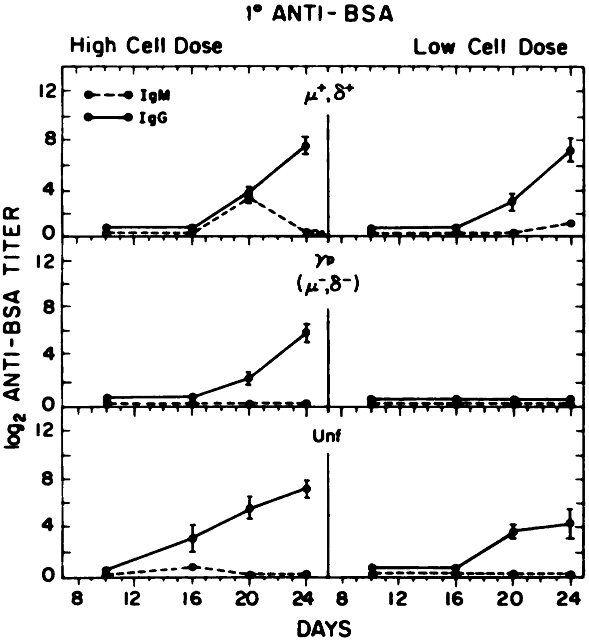

FIG. 9. Adoptive primary anti-BSA response restored by (μ^+ + δ^+) spleen cells. Cells were stained with a combination of RAμ and RAδ, and separated on the FACS. Irradiated mice received a dose of bright (μ^+ + δ^+) or dull (μ^-, δ^-) cells contained in 4 or 0.8 × 10^6 unfractionated cells. All recipients were given 5 × 10^6 unprimed T cells. Controls received 4 or 0.8 × 10^6 unfractionated cells plus T cells or T cells alone. △, 0.03 × 10^6 (μ^+ + δ^+) cells (dose of bright cells contaminating dulls); □, T cells alone. Each points represents the mean response of a group of four to eight mice, and brackets show the standard error.

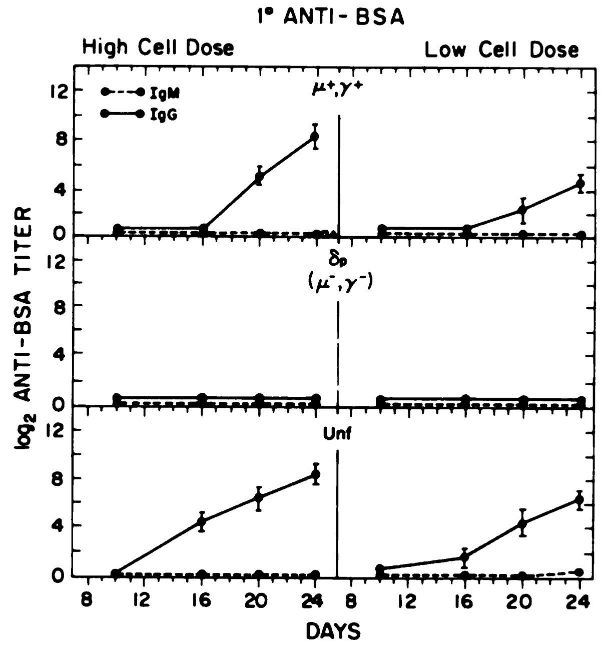

FIG. 10. Adoptive primary anti-BSA response restored by ($\mu^+ + \gamma^+$) spleen cells. Irradiated mice received a dose of bright ($\mu^+ + \gamma^+$) or dull (μ^-, γ^-) cells contained within 4 or 0.8 × 10^6 unfractionated cells. All recipients were given 5 × 10^6 unprimed T cells. △, 0.06 × 10^6 ($\mu^+ + \gamma^+$) cells (dose of bright cells contaminating dulls); □, T cells alone. Each point represents the mean of a group of four to eight mice, and brackets show the standard error.

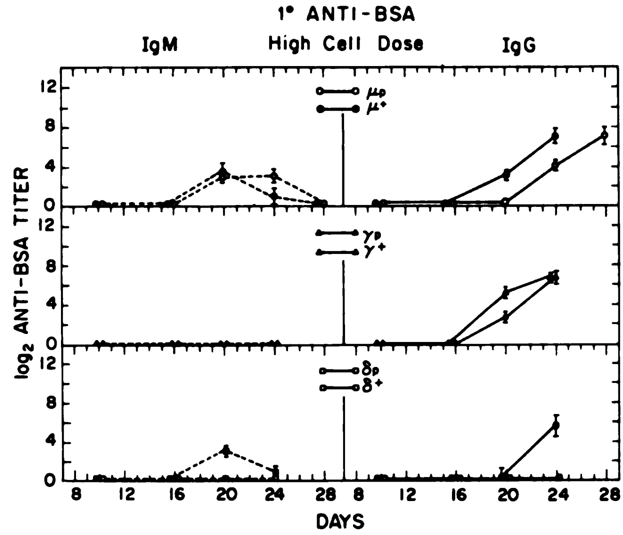

FIG. 11. Comparison of the adoptive primary anti-BSA responses restored by isotype-positive and isotype-predominant cells (high cell dose). Isotype-positive cells are in the bright cell fraction obtained after staining with a single anti-isotype antiserum. Isotype-predominant cells are in the dull cell fraction obtained after staining with two anti-isotype antisera. Irradiated recipients received a dose of μ^+, δ^+, γ^+, μp, δp, or γp cells contained within 4 × 10^6 unfractionated cells. All recipients were given 5 × 10^6 unprimed T cells. Each point represents the mean of a group of four to eight mice, and brackets show the standard error.

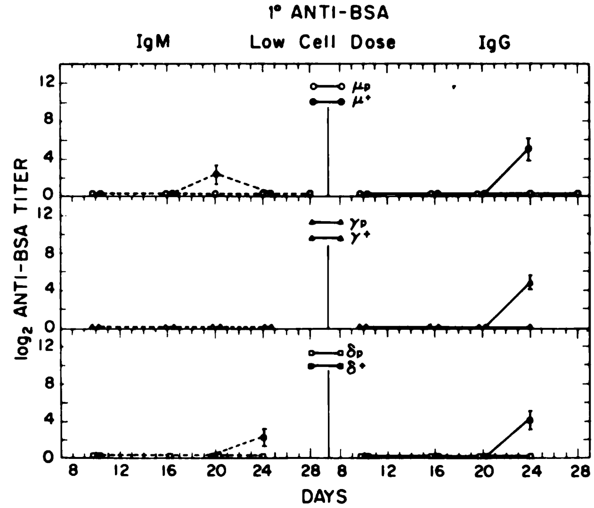

FIG. 12. Comparison of the adoptive primary anti-BSA responses restored by isotype-positive and isotype-predominant cells (low cell dose). Irradiated recipients received a dose of μ^+, δ^+, γ^+, μp, δp, or γp cells contained within 0.8×10^6 unfractionated cells. All recipients were given 5×10^6 unprimed T cells. Each point represents the mean of a group of four to eight mice, and brackets show the standard error.

Comparison of the Primary Anti-BSA Response Restored by Isotype-Positive and Isotype-Predominant Cells. Fig. 11 compares the IgM and IgG responses restored by isotype-positive and isotype-predominant cells at the high cell dose. Although the μ^+ and μp cells produced both an adoptive IgM and IgG response, the switch to IgG synthesis with the latter cells was delayed by 4 days with respect to the former. This suggests that the μp cell is a less mature B cell than the $\mu^+\delta^+$ cell and, therefore, requires further maturation in the adoptively transferred host before giving rise to the IgG response. The γ^+ and γp cells produced similar adoptive responses which were all IgG. The most dramatic difference between isotype-positive and predominant cells was observed with the δ^+ and δp cells. The former subpopulation restored both the IgM and IgG response, but the latter produced no detectable antibody at the high cell dose. These results suggest that the IgM-IgD double bearers are the major precursors of the primary IgM response but that these cells do not undergo significant differentiation into δp cells in the absence of antigen.

Fig. 12 shows that the adoptive responses restored by isotype-positive cells were considerably reduced at the low cell dose. However, none of the isotype-predominant cells produced detectable responses at the same dose. This shows that the contribution of isotype-predominant cells to the adoptive primary response is less than that of isotype-positive cells.

Discussion

The object of these experiments was to identify B cells in the mouse spleen that bear a predominant Ig isotype and to determine the functional activity of these cells in the adoptive primary and secondary anti-BSA responses. To isolate and examine such putative isotype-predominant B cells, we first stained spleen cells for all three major surface isotypes (μ, δ, γ) simultaneously and separated the bright (positively staining) and dull (negatively staining) cells on the FACS. The restorative activity of the bright cells was similar to unfractionated cells, but no detectable activity was observed with the dull cells. In subsequent experiments, spleen cells were stained for two of the three surface isotypes, and the restorative activity of the dull cells was examined. Adoptive responses produced by such dull cells were attributed to the presence of B cells bearing only the remaining surface isotype which was not detected by the staining procedure.

Comparisons were made between the responses restored by isotype-positive cells (bright cells isolated by the FACS after staining spleen cells for a single surface isotype) and those restored by isotype-predominant cells. The isotype-positive cells include all cells staining positively for a given isotype, and include isotype-predominant cells as well as those cells that bear that isotype in combination with others. The results show that the contribution of isotype-predominant cells to the adoptive primary and secondary responses restored by a given number of spleen cells is at least five times smaller than that of the isotype-positive cells. This suggests that the large majority of B cells bearing a given surface isotype express that isotype in combination with others. This finding is consistent with the considerably greater percentage of isotype-positive as compared with isotype-predominant cells observed in the spleen by direct analysis of stained cells on the FACS. For example, the mean percentage of δ^+ cells in spleen cells was 43% (8, 9), and the calculated percentage of δp cells in the present experiments was 3-4%. Similarly determined values for μ^+ and μp are 35 and 2-3%, respectively, and for γ^+ and γp are 13% and 0.5-1% (8, 9), respectively.

The experimental findings show that μp, δp, and γp cells are all active in restoring the adoptive secondary anti-BSA response and, therefore, carry immunological memory. The response produced by μp cells is almost all IgM at day 7, and switches to IgG antibody (2-ME resistant) by day 9. On the other hand, both the δp and γp cells restore only an IgG response with rapid kinetics so that day 7 titers are already approaching the plateau levels. The μp cells may not be typical long-lived, recirculating memory cells, but rather, recently activated virgin B cells which are their immediate precursors. Recent studies in rats have shown that IgM-bearing blast cells that carry immunological memory are found in the thoracic duct lymph for several weeks after priming with antigen in complete Freund's adjuvant (23).

The kinetics and magnitude of the secondary anti-BSA response restored by μ^+ and μp cells on the one hand, and by γ^+ and γp cells on the other, were similar at the high cell dose (equivalent of 2×10^6 cells). The IgG antibody produced by the μp cells could not be accounted for by the contaminating (γ^+ + δ^+) cells because the transfer of a dose of (γ^+ + δ^+) cells that represents the maximum number that could have contaminated the μp inoculum does not produce a detectable IgG response. In addition, the (μ^-, δ^-, γ^-) cells, which should have the same γ^+ contamination as the (γ^-, δ^-) cells, do not restore an adoptive response.

These findings strongly suggest that only B cells bearing surface IgM (alone or in combination with other isotypes) give rise to IgM secreting progeny. This interpretation is consistent with our own previous results (8, 9), the results of Pierce et al. (1, 2), Yuan et al. (5), Coffman and Cohn (6), and Cambier et al. (10). However, our finding that μp cells give rise to IgG secreting progeny is more controversial because several laboratories have reported that only IgG-bearing cells give rise to the adoptive secondary IgG response (3, 4). Some of the differences may be reconciled by the differences in the kinetics of the IgG response restored by different B-cell subpopulations. For example, IgG-positive cells give rise to a rapid IgG response approaching the plateau on day 7. On the other hand, IgM-positive or IgM-predominant cells give rise to a 7-day response that is almost all IgM, and only produces a substantial IgG response at day 9. Measurement of the early indirect plaque-forming cell response at only one time-point would suggest that only the γ^+ cells carry immunological memory.

The response restored by the δ^+ cells differed from that of the δp cells at this dose, in that the former generated first an IgM and then an IgG response, whereas the latter generated only an IgG response. This suggests that cells bearing both IgM and IgD give rise to progeny that secrete first IgM and then IgG. In contrast, δp cells (which are μ^-) give rise to progeny that secrete only IgG. This observation is consistent with a model of B-cell differentiation suggested previously (15). Thus, as IgM-IgD double bearers are stimulated by antigen they may lose surface IgM and become δp cells. The δp cells are committed to IgG synthesis. Although the δp cells are a minor subpopulation of B cells in the spleen, these cells appear to make up the majority of B cells in the thoracic duct lymph (24).

Regulatory interactions between subpopulations of B cells were observed in experiments in which μ^+ and/or δ^+ cells were mixed with γ^+ or γp cells. Vigorous adoptive IgM responses were restored by μ^+, δ^+, μp cells, or a combination of μ^+ and δ^+ cells. However, *no* IgM response was produced by combinations of ($\mu^+ + \gamma^+$), ($\delta^+ + \gamma^+$), or ($\mu^+ + \delta^+ + \gamma^+$) cells. This shows that γ^+ cells or their products suppress the IgM response of the μ^+ and δ^+ cells. It is likely that only the γp cells within the γ^+ population are capable of suppressing the IgM response, because the combination ($\mu^+ + \delta^+$) cells gives rise to IgM antibody, and this population lacks only the γp subpopulation as compared to ($\mu^+ + \delta^+ + \gamma^+$) cells. The γp cells maintain their suppressive activity even at the low doses at which they are unable to restore a detectable IgG response. Thus, ($\mu^+ + \gamma^+ + \delta^+$) cells produce only IgG antibody, ($\mu^+ + \delta^+$) cells produce both IgM and IgG antibody, and γp cells produce no detectable antibody at the low cell dose. The findings raise the possibility that the mechanism of suppression may occur via cell-cell interaction rather than by serum IgG antibody. However, we have not excluded the possibility that low levels of serum antibody not detectable by the procedures employed are responsible for the inhibition. It is interesting to note that the δp cells do not appear to suppress the IgM response of μ^+ cells, despite the fact that δp cells give rise only to IgG antibody. The difference in the ability of the two subsets to suppress the IgM response may be due to the affinity of the antibody they produce. Thus a small amount of high affinity IgG produced by the γp cells may adequately suppress the IgM response. In contrast, δp cells may give rise to lower affinity antibody which, when present in small amounts, is not suppressive.

The results of our studies of the adoptive primary anti-BSA response were similar to those of the adoptive secondary. Unprimed μp cells restored the adoptive primary IgM and IgG responses, but unprimed γp cells restored only the IgG response. The contribution of μp and γp cells to the adoptive primary response was considerably smaller than that of the μ^+ and γ^+ cells (isotype-positive cells), because only the latter cells made a detectable response at the low cell dose (equivalent of 0.8×10^6 cells). The anti-BSA response of the μp cells differed from that of the μ^+ cells in that the switch from IgM to IgG antibody was delayed by about 4 days. These results suggest that the μp cells are a less differentiated population of cells than the majority of the μ^+ cells (which are μ^+ δ^+ doubles). The existence of μp cells has been postulated previously, and it is possible that these cells are the precursors of the μ^+ δ^+ cells. As in the adoptive secondary response, the γp cells suppressed the IgM response of the μ^+ and δ^+ cells. This is shown by a comparison of the adoptive primary anti-BSA responses restored by (μ^+ + δ^+) cells and (μ^+ + δ^+ + γ^+) cells. The latter cells include the former, and in addition contain γp cells. Whereas the (μ^+ + δ^+) cells produce an adoptive response that is almost all IgM at day 20, the (μ^+ + δ^+ + γ^+) produce a response of similar magnitude that is mainly IgG.

The role of γ^+ and γp cells in the adoptive primary response remains to be elucidated. The cells could be the product of prior exposure of the normal (BALB/c × C57BL/Ka)F_1 mice to environmental antigens that cross react with determinants expressed by BSA. On the other hand, these subpopulations may be derived from B-cell precursors via an antigen-independent maturation sequence as suggested by Cooper and his colleagues (25). In that case, the γp or γ^+ cells may be members of a separate line of B cells that mature parallel with the μp and $\mu^+\delta^+$ cells.

The ability of the unprimed μp cells to give rise to IgG antibody extends and confirms our earlier observation that B cells that do not stain positively for surface IgG can, nevertheless, transfer an adoptive primary IgG response. Contamination of μp cells staining positively for surface IgG cannot account for the adoptive IgG response because the transfer of a dose of IgG-positive cells equivalent to the maximum number that could contaminate the μp cells does not produce a detectable adoptive primary IgG response. In addition, the (μ^-, δ^-, γ^-) cells, which should have the same γ^+ contamination as the γ^-, δ^-) (μp) cells, do not restore an adoptive response. The present study did not determine whether μp cells must express a small quantity of surface IgG below the levels of detection by fluorescence microscopy, or whether the cells acquire surface IgG during residence in the adoptive host.

The inability of δp cells to transfer the adoptive primary anti-BSA response contrasts with the considerable restorative activity of μp and γp cells in the primary response, and δp cells in the secondary response. This suggests that functional δp cells are present in very small numbers, if at all, among unprimed B cells, but are present in much larger quantities relative to all other B-cell subpopulations after priming. Indeed, it is possible that significant restorative activity in the δp subpopulation of a given mouse is a marker for prior exposure to the antigenic determinants under investigation. Because the relationship between the two IgG precursors (δp and γp) is not clear at present, it is difficult to speculate on the presence of the latter but not the former in unprimed mice. It is possible that the two cells are derived as separate lineages or that the accumulation of γp cells is less antigen dependent than that of the δp cells. In addition, there may be unique regulatory mechanisms in unprimed mice that allow the accumulation of γp but not δp cells.

The placement of the isotype-predominant cells in the scheme of antigen-independent and antigen-dependent B-cell maturation remains speculative. However, a model consistent with our experimental findings would place B-cell precursors bearing only surface IgM in the neonatal spleen and adult bone marrow. These cells may give rise to the unprimed B cells in the adult spleen which include populations of μ^+, δ^+, and γ^+ cells. All three populations make a considerable contribution to the adoptive primary response. The μp and γp cells are minor subpopulations contained within the set of μ^+ and γ^+ cells. It is possible that the γ^+ set present in unprimed mice are the result of stimulation of μ^+ and δ^+ cells with environmental antigens that cross react with the determinants under investigation. On the other hand, these cells may be members of a lineage that develops from the young μp cells via antigen-independent maturation in parallel with the (μ^+, δ^+) cells.

This general maturation scheme may be recapitulated after antigenic stimulation. Primed μp cells may represent recently activated virgin B cells that differentiate into (μ^+, δ^+) and γ^+ memory cells. On the other hand, γ^+ memory cells may arise in parallel to (μ^+, δ^+) memory cells from γ^+ virgin cells.

Summary

We determined whether primed and unprimed B cells in the spleen of (BALB/c × C57BL/Ka)F_1 mice contain subpopulations that express a predominant surface Ig isotype. Spleen cells were stained for surface isotypes and sorted on the fluorescence-activated cell sorter (FACS) in order to obtain B cells bearing predominantly IgM (μp cells), IgD (δp cells), or IgG (γp cells). Each population was assayed for its capacity to restore the adoptive primary and secondary anti-bovine serum albumin (BSA) antibody response in irradiated syngeneic recipients. In addition, the adoptive response restored by isotype-predominant cells was compared to that restored by isotype-positive cells (B cells bearing a given surface isotype alone or in combination with others). The experimental results show that μp cells restore the adoptive primary and secondary IgM and IgG responses to BSA, and γp cells restore only the primary and secondary IgG response. δp Cells restored the adoptive secondary IgG response, but failed to restore the adoptive primary response at the cell doses tested. γp Cells but not δp cells suppressed the IgM response of the μ^+ and δ^+ cells. The contribution of isotype-predominant cells to both the adoptive primary and secondary anti-BSA response was smaller than that of B cells bearing a combination of surface isotypes. Differences in the Ig isotype pattern expressed on the surface of primed and unprimed B cells are discussed.

We knowledge the expert technical assistance of Ms. M. Bagby, Mr. Y. Chinn, Mr. S. Lin, Ms. G. Sloan, Mr. M. Knapp, Ms. C. Doss, and Ms. J. Terry. We thank Ms. M. Janisch and Ms. J. Hahn for secretarial assistance.

Received for publication 28 October 1977.

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T-LYMPHOCYTE RESPONSE TO *H-2* MUTANTS

I. Proliferation is Dependent on Ly $1^{+}2^{+}$ Cells*

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Lymphocyte differentiation (Ly)[1] antigens serve as useful markers for distinguishing thymus-derived (T) lymphocyte subpopulations. Initially, Boyse and co-workers (1, 2) discovered the Ly 1 and Ly 2, 3 alloantigen systems, expressed on partially overlapping subsets of T lymphocytes. More recently the number of known Ly antigen systems has expanded with antigens expressed on subpopulations of T lymphocytes (3, 4), B lymphocytes (5–7), and B and T lymphocytes (8). Boyse and Old proposed early on that Ly antigens are expressed differentially on lymphocyte subpopulations which have diverged in immunological function (9). Recently this proposal has been confirmed by Cantor and Boyse (10, 11) who distinguished lymphocytes responsive to major histocompatibility (H-2) complex antigens in mixed lymphocyte culture (MLC) and cell-mediated lympholysis (CML) on the basis of Ly 1, 2, and 3 phenotypes. Responder cells in MLC were Ly $1^{+}2^{-}3^{-}$ T lymphocytes whose proliferation was required for the generation of Ly $1^{-}2^{+}3^{+}$ effector cells detectable in CML. However, it is not clear whether the responsive lymphocyte subpopulations are specific for the assayed functions, proliferation versus cytolysis, or specific for the different target alloantigens, I region-associated (Ia) alloantigens in MLC (12) versus H-2K and H-2D alloantigens in CML (13).

To understand more fully the significance of the differential responses of Ly $1^{+}2^{-}3^{-}$ and Ly $1^{-}2^{+}3^{+}$ lymphocytes, it is particularly important to elucidate the basis for these differential responses. We wished to determine if the subpopulation of lymphocytes responsive to a class of determinants were dependent on the antigenic determinant, Ia or K/D, rather than on their immunologic function, proliferation or cytolysis. *H-2* mutants provide an opportunity to study both proliferation of responders and the generation of cytotoxic effectors to single antigenic determinants since mutations in *H-2K* and *H-2D* region genes have generated novel antigenic specificities detectable in both MLC and CML (14–16). In this communication we report observations made in Ly typing T lymphocytes proliferating in response to mutant H-2K and H-2D antigens in

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* Supported by American Cancer Society grant IM-90, National Science Foundation grant PCM-77-02772

‡ Leukemia Society of America Postdoctoral Fellow.

[1] *Abbreviations used in this paper:* C, complement, CML, cell-mediated lympholysis; FCS, fetal calf serum; H, histocompatibility; Ly, lymphocyte; MLC, mixed lymphocyte culture; NMS, normal mouse serum.

primary MLC. Proliferating T lymphocytes responsive to mutant H-2K^{ba} and H-2K^{bf} antigens were classified as Ly 1$^+$2$^+$ lymphocytes by selective depletion analysis. The implications of these results for our understanding of T-lymphocyte differentiation and maturation are discussed.

Materials and Methods

Mice. The mice employed in this study and their respective genotypes are presented in Table I. The mutant *H-2*^{ba} and *H-2*^{bf} haplotypes of the B6.C-*H-2*^{ba} By (B6-*H-2*^{ba}) and B6.C-*H-2*^{bf} By (B6-*H-2*^{bf}) mice were discovered by Bailey and Kohn through reciprocal circle grafting analyses designed to identify histocompatibility (*H*) gene mutations (17). Complementation tests demonstrated that both mutations occurred in the *K* end of the *H-2*^{b} haplotype (18, 19). Reciprocal, primary MLC of B6-*H-2*^{ba} and B6-*H-2*^{bf} lymphocytes and B6 lymphocytes result in responder proliferation (14, 19). The *H-2*^{da} haplotype of B10.D2 (M504) (B10.D2-*H-2*^{da}) was discovered by Egorov and collaborators to have diverged from the *H-2*^{d} haplotype of B10.D2/n by a mutation in the *D* region of *H-2*^{d} (20). B10.D2-*H-2*^{da} and B10.D2/n lymphocytes are reciprocally stimulatory in primary MLC (15, 16). Mice of the B6-*Ly-1*^{a} strain were selected to be congenic with, but differ from, C57BL/6 (B6) mice at the *Ly 1* locus (21). B6-*Ly 1*^{a} mice are *Ly 1*^{a} whereas B6 mice are *Ly 1*^{b}. Both strains share the *Ly 2*^{b}, *3*^{b} genotype. According to genetic convention (22), *Ly 1*^{a} and *Ly 1*^{b} alleles determine the Ly 1.1 and Ly 1.2 alloantigens, respectively.

Antisera. Anti-Ly and anti-Thy 1 sera were raised by immunization of recipients with thymocytes obtained from congenic donors differing at the locus of interest. The cell dosage and schedule of immunization and bleeding have been described previously (23). Anti-Ly 1.1 serum was produced by immunization of (BALB/c × C57BL/10)F$_1$ recipients with B6-*Ly 1*^{a} thymocytes. Anti-Ly 2.2 serum was prepared by immunization of (C3H.OL × B6-Ly 2^{a})F$_1$ hosts with C57BL/6 thymocytes. Individual sera were tested as suggested by Shen and co-workers (24); only active antisera were pooled. Anti-Thy 1.2 serum was produced by immunization of A.AKR(*Thy 1*^{a},*H-2*^{a1}) mice with A.AL (*Thy 1*^{b}, *H-2*^{a1}) thymus cells. Specificity tests in complement-dependent cytotoxicity assays demonstrated that this anti-Thy 1.2 serum is specific for T cells from *Thy 1*^{b} donors (J. A. Frelinger, unpublished observations).

Complement-Dependent Cytotoxicity Testing. The alloantigenic specificity of all alloantisera were tested in a complement-dependent cytotoxicity assay described by David et al. (23). Selected young rabbit serum obtained from Pel-Freeze Farms, Inc., (Rogers, Ark.) served as the source of complement for tests with spleen lymphocyte targets.

Selective Lymphocyte Depletion. Lymphocytes bearing selectively expressed cell-surface alloantigens were eliminated from lymphocyte populations as described by Niederhuber et al. (25). Briefly, lymphocytes were suspended in alloantiserum diluted in RPMI-1640 (Microbiological Associates Walkersville, Md.) + heat-inactivated fetal calf serum (FCS) at a concentration of 5% at 10 × 10^{6} cells/ml diluted antiserum. The antiserum-treated lymphocytes were incubated at 37°C for 30 min, centrifuged, and resuspended in rabbit complement appropriately diluted in RPMI-1640 at 10 × 10^{6} cells/ml diluted complement. After incubation at 37°C for 40 min, the surviving lymphocytes were resuspended in RPMI-1640 + 5% FCS and washed three times in RPMI-1640.

Mixed Lymphocyte Culture. The MLC was performed as a modification of the procedure described by Peck and Bach (26) as we have described in a previous communication (27). Briefly, responder cells were T lymphocytes enriched from Tris-ammonium chloride-treated spleen cells by passage through nylon wool columns (27). After nylon wool passage, responder T cells were employed in MLC either without further treatment or after selective lymphocyte depletion with antiserum plus complement. Stimulator cells were Tris-ammonium chloride-treated spleen cells inactivated by mitomycin C (Sigma Chemical Co., St. Louis, Mo.) treatment. A total of one million responder and stimulator cells were mixed in either a 1:1 or 1:3 ratio in quadruplicate cultures in Click's medium (29) without nucleic acid precursors but supplemented with Hepes (30 mM) and normal mouse serum (1%). The cultures were incubated for 5 days at 37°C in humidified Lucite boxes with an atmosphere of 83% nitrogen, 10% carbon dioxide, and 7% oxygen. 24 h before harvest the cultures were pulsed with [^{3}H]thymidine (New England Nuclear, Boston, Mass. 2 Ci/mM) at 2 μCi/well. The cultures were harvested and [^{3}H]thymidine uptake determined as described previously (27). Specific [^{3}H]thymidine uptake was calculated as a Δ when the difference

TABLE I

H-2 Haplotypes and Ly 1,2 Genotypes of Employed Mouse Strains

Strain	*H*-2 Haplotype	Origin of *H-2* regions				*Ly 1*	*Ly 2*
		K	*I*	*S*	*D*		
C57BL/6-*Ly* 1^a	*b*	*b*	*b*	*b*	*b*	*a*	*b*
C57BL/6-*H-2*^{ba}	*ba*	*ba*	*b*	*b*	*b*	*b*	*b*
C57BL/6-*H-2*^{bf}	*bf*	*bf*	*b*	*b*	*b*	*b*	*b*
B10.D2/n	*d*	*d*	*d*	*d*	*d*	*b*	*b*
B10.D2(M504) (B10.D2-*H-2*^{da})	*da*	*d*	*d*	*d*	*da*	*b*	*b*
B10	*b*	*b*	*b*	*b*	*b*	*b*	*b*
B10.S	*s*	*s*	*s*	*s*	*s*	*b*	*b*
B10.A(1R)	*h1*	*k*	*k/d*	*d*	*b*	*b*	*b*
B10.A(2R)	*h2*	*k*	*k/d*	*d*	*b*	*b*	*b*
B10.C-*H-7*^{b}	*b*	*b*	*b*	*b*	*b*	*b*	*b*
C3H.OL	*ol*	*d*	*d*	*k*	*k*	*a*	*a*
C3H.Q	*q*	*q*	*q*	*q*	*q*	*a*	*a*

between mean uptake in allogenic and syngeneic cultures differed at the $\alpha = 0.001$ level according to Student's *t* test assuming equal variance (30).

Cell-Mediated Lympholysis. The CML assay was performed according to the technique described by Hirschberg et al. (31). All incubations and washes were performed in RPMI-1640 supplemented with heat-inactivated FCS (10%), gentamycin (0.5%) (Schering Diagnostics, Kenilworth, N. J.) and 2-mercaptoethanol (10^{-5} mM). Target cells were lymphoblasts which had been generated by incubation of splenic lymphocytes with concanavalin A (2 μg ConA/2 × 10^6 cells/ml) (Calbiochem, San Diego, Calif.) for 2–3 days. Viable lymphoblasts were separated by flotation on Ficoll-Hypaque (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) followed by washes at 50 *g*. Lymphoblasts were labeled with ^{51}Cr (Amersham Corp., Arlington Heights, Ill.), at 200 μCi/2 × 10^6 cells for 60 min, washed four times, and suspended to 2 × 10^5 cells/ml. Effector cells were generated in MLC under conditions and cell concentrations identical to those employed in microculture as described above with the exception that 5 ml of both responder and stimulator cells were mixed in 25 cm^2 tissue culture flasks. Effector cells were harvested after 6–7 days of culture. Effectors were washed twice, counted, and resuspended. 100 μl of the target and effector suspensions were mixed in quadruplicate in U bottom wells of microtiter plates (Flow Laboratories, Inc., Rockville, Md.). The plates were centrifuged for 5 min at 50 *g*. The cultures were incubated for 4 h in an atmosphere of 83% N_2 10% CO_2, and 7% O_2 in humidified Lucite boxes at 37°C. After incubation the supernates were collected with the aid of a Skatron supernatant collection system (Flow Laboratories). Total releasable label was the ^{51}Cr released after two cycles of freeze-thawing. The specific ^{51}Cr release was determined by the following equation:

percent specific release = experimental release − spontaneous release
/total releasable label-spontaneous release × 100.

Results

Ly Antisera Characterization. To insure the correct specificity of the anti-Ly 1.1 and anti-Ly 2.2 sera employed in this study, specificity tests were performed in complement-dependent cytotoxicity, MLC, and CML. The alloantigenic specificity of anti-Ly 1.1 and anti-Ly 2.2 sera were tested with B6-*Ly* 1^a and B6-*Ly* 2^a splenic lymphocytes in the complement-dependent cytotoxic test. The results of this assay are presented in Fig. 1. Both antisera were strongly positive on B6-*Ly* 1^a spleen cells and negative on B6-*Ly* 2^a lymphocytes, demonstrating that both sera were specific for their appropriate alloantigens.

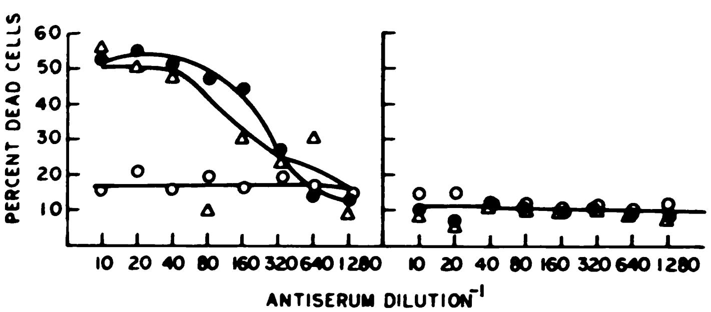

FIG. 1. Complement-dependent dye exclusion cytotoxic test of anti-Ly 1.1 and anti-Ly 2.2. Left panel sera tested on B6-*Ly 1*^{a} (Ly 1.1 and Ly 2.2) spleen cell targets, (○——○ NMS; △——△ anti-Ly 1.1; ●——● anti-Ly 2.2). Right panel, sera tested on B6-*Ly 2*^{a} (Ly 1.2 and Ly 2.1). Same symbols as left.

TABLE II

Specificity of Anti-Ly Sera for Selective Depletion of Responder T Cells in MLC

Responders	Ly 1, 2 Phenotype	Stimulator	Antiserum + C		Inhibition‡
				Δ cpm*	%
B6-Ly *1*^{a}	Ly 1.1, 2.2	B10.D2	NMS (1/40)	72,180	43
			α-Ly 1.1 (1/40)	45,768	
B10.S	Ly 1.2, 2/2	B10.D2	NMS (1/40)	114,011	
			α-Ly 1.1 (1-40)	102,512	10
C3H.Q	Ly 1.1, 2.1	C3H.OL	NMS (1/20)	211,298	
			α-Ly 1.1 (1/20)	25,470	88
B10.S	Ly 1.2, 2.2	B10.D2	NMS (1/20)	140,503	
			α-Ly 2.2 (1/20)	164,248	−17
B10	Ly 1.2, 2.2	B10.A (2R)	NMS (1/10)	129,383	
			α-Ly 2.2 (1/10)	112,055	13

* Δ = Mean [^{3}H]thymidine uptake for allogeneic combination − mean [^{3}H]thymidine uptake for syngeneic combination. Computed when the two means differ at $\alpha \leq 0.001$.

‡ Inhibition = (Δ after alloantiserum + C treatment/Δ after normal mouse serum + C treatment) × 100.

The slight degree of nonspecific lysis observed in the complement-dependent cytotoxic test does not necessarily indicate that a similar lack of specificity will be observed in depletion of T-lymphocyte function, due to the greater sensitivity of the analytical dye exclusion cytotoxic test. Both the alloantigenic and T-cell subpopulation specificity of the Ly antisera were tested by pretreatment of MLC responder T cells and CML effector T cells with antisera plus complement. Responder and stimulator cells mixed in MLC were incompatible at the *I* region, *K* region, and/or *D* region. Nylon wool T cells obtained from *Ly 1*^{a} and *Ly 1*^{b} donors were treated with anti-Ly 1.1 serum or normal mouse serum (NMS) + complement (C). The results are presented in Table II. Relative to the effects of NMS + C, anti-Ly 1.1 + C depleted the proliferative response of Ly 1.1-positive responder cells (B6-*Ly 1*^{a} and C3H.OL) with no effect on the

TABLE III

Depletion of H-2-Specific Cytotoxic Effector Cells with Anti-Ly 2.2 Serum

Effector cell donor	Ly 1, 2 Phenotype	Target	Antiserum + C	% ^{51}Cr Release*	Inhibition‡
					%
(B10.C-*H-7*^{b} × C3H.OL)F$_1$	Ly 1.1/1.2 Ly 2.1/2.2	C3H.Q	–	60.8 ± 3.8	
			NMS (1/20)	50.2 ± 4.2	
			α-Ly 1.1 (1/20)	41.2 ± 1.9	17.6
			α-Ly 2.2 (1/20)	0§	100.0
		B10.C-*H-7*^{b}	–	0	
			NMS (1/20)	0	
			α-Ly 1.1 (1/20)	0	
			α-Ly 2.2 (1/20)	0	

* Effector: target ratio of 20:1.

‡ Inhibition = (^{51}Cr release after antiserum + C/^{51}Cr release after NMS + C) × 100.

§ ^{51}Cr release reported as zero when value is less than or equal to zero.

TABLE IV

Ly 2 Typing of T Cells Proliferating in MLC to H-2D Alloantigen Determined by the H-2D^d: H-2D^{da} Allelic Combination

Responder	Ly 2 Phenotype	Stimulator	Target antigens	Antiserum + C		Inhibition‡
					Δ cpm*	%
B10.D2	Ly 2.2	B10.D2-*H-2*^{da}	H-2D^{da}	–	151,222	
				NMS	152,462	-1
				α-Thy 1.2	0	100
				α-Ly 2.2	12,337	92
B10.D2-*H-2*^{da}	Ly 2.2	B10.D2	H-2D^d	–	24,284	
				NMS	10,772	56
				α-Thy 1.2	0	100
				α-Ly 2.2	2,548	90

* As in Table II.

‡ As in Table II.

response of Ly 1.1-negative responders (B10.S). Therefore, the anti-Ly 1.1 serum was specific for the Ly 1.1 alloantigen on T cells proliferating in primary MLC. The T-cell subpopulation specificity of anti-Ly 2.2 serum was determined by treating nylon wool T cells obtained from *Ly 2*^{b} donors with anti-Ly 2.2 serum + C before culture with H-2-incompatible stimulators. The results of this assay are included in Table II. Anti-Ly 2.2 + C did not deplete B10.S and B10 T cells responsive in MLC to B10.D2 and B10.A(2R) stimulators. Anti-Ly 2.2 but not anti-Ly 1.1 serum plus complement depleted (C3H.OL × B10.C-H-7^b)F$_1$ effectors specific for C3H.Q targets (Table III). These observations are consistent with previous reports in demonstrating that T lymphocytes proliferating in response to H-2-incompatible stimulators in MLC are Ly 1$^+$2$^-$ whereas the cytotoxic effectors specific for H-2-alloantigens are Ly 1$^-$2$^+$ (10, 11).

Ly Typing of H-2 Mutant Antigen-Responsive T Lymphocytes. We Ly 2 typed the T cells proliferating in reciprocal primary MLCs of B10.D2/n and B10.D2-H-2da lymphocytes. The results of this experiment are presented in Table IV. Relative to the inhibition observed with NMS + C, anti-Ly 2.2 + C

TABLE V

Ly Typing of T Cells Proliferating in MLC to H-2K^{ba} and H-2K^{bf} Mutant Alloantigens

Responder	Ly 1, 2 Phenotype	Stimulator	Target antigen	Antiserum + C		Inhibition‡
					Δ cpm*	%
B6-Ly 1^a	Ly 1.1, 2.2	B6-*H-2*^{ba}	H-2K^{ba}	–	49,422	
				NMS	51,268	
				α-Thy 1.2	0	100
				α-Ly 1.1	0	100
				α-Ly 2.2	1,597	97
				α-Ly 1.1	699	99
				α-Ly 1.1 treated cells mixed with α-Ly 2.2-treated cells		
		B6-*H-2*^{bf}	H-2K^{b*}	–	13,325	
				NMS	5,866	
				α-Thy 1.2	0	100
				α-Ly 1.1	0	100
				α-Ly 2.2	0	100
				α-Ly 1.1-	0	100
				α-Ly 1.1 treated cells mixed with α-Ly 2.2-treated cells		
		B10.A(1R)	H-2K^{k}I$^{k/d}$	–	NT	
				NMS	124,857	
				α-Thy 1.2	2,065	98
				α-Ly 1.1	2,843	98
				α-Ly 2.2	97,335	22.1
				α-Ly 1.1-treated cells mixed with α-Ly 2.2-treated cells	50,030	60

* As in Table II.

‡ As in Table II.

and anti-Thy 1.2 + C eliminated the T cells responsive in reciprocal MLCs. This observation strongly indicated that T lymphocytes proliferating in response to the antigens defined by the H-2D^d: H-2D^{da} responder: stimulator combination are Ly 2$^+$. Although we did not have the anti-Ly 1.2 sera available to Ly 1 type these responders it is clear that these proliferating T cells expressed a different phenotype than Ly 1$^+$2$^-$ responders in *I*-region disparate MLC.

A more definitive Ly typing of H-2K mutant antigen-responsive T cells was accomplished through the use of anti-Ly 1.1 and anti-Ly 2.2 sera and B6-*Ly 1*^{a} T cells. B6-*Ly 1*^{a} T cells were pretreated with anti-Ly 1.1 or anti-Ly 2.1 + C and mixd in primary MLC with mitomycin C-treated spleen cells from B6-*H-2*^{ba}, B6-*H-2* ^{bf}, and B10.A(1R) mice. The results are presented in Table V. B6-*Ly 1*^{a} mice are congenic with the Boyse substrain of B6, differing at a segment of chromosome encompassing the *Ly 1* locus. The long separation of the B6/Boy and B6/By (background strain of B6-*H-2*^{ba} and B6-*H-2*^{bf} congenic strains) suggests that there is a high probability that the two substrains differ by one or more *H* loci. However, primary cultures of B6-*Ly 1*^{a} responder cells and B6/By stimulator cells do not result in detectable proliferation. We are confident that the observed proliferation of B6-*Ly 1*^{a} responders mixed with B6-*H-2*^{ba} and B6-*H-2*^{bf} stimulators is specific for H-2K^{ba} and H-2K^{bf} alloantigens. Responders to both mutant stimulators and B10.A(2R) stimulators were depleted by anti-Thy 1.2 and anti-Ly 1.1 serum plus C. The crucial experiment was pretreatment with anti-Ly 2.2 + C. This treatment resulted in depletion of the T-cell response to both B6-*H-2*^{ba} and B6-*H-2*^{bf} stimulators. The same treated cells responded normally to B10.A(1R) stimulators. These results intimated that proliferation in MLC to H-2K-incompatible stimulators in contrast to *I* region-incompatible stimulators was mediated by Ly 1^{+}2^{+} T cells or required cooperation of Ly 1^{+}2^{-} and Ly 1^{-}2^{+} T cells. To discriminate between these two alternatives, we tested the ability of mixtures of T cells depleted of Ly 1^{+} cells and T cells depleted of Ly 2^{+} cells to respond to mutant stimulators. These results are included in Table V. Mixtures of Ly 1^{-} and Ly 2^{-} T cells were unresponsive to both *H-2K*^{b} mutant stimulators indicating that the lymphocytes responding to H-2K^{ba} and H-2K^{bf} antigens were Ly 1^{+}2^{+}.

Discussion

Lymphocyte differentiation antigens have long been believed to be expressed differentially on lymphocyte subpopulations which have diverged functionally during development and maturation. This supposition has been confirmed by the demonstration that T cells proliferating in primary MLC in response to *H-2K* plus *I* region gene coded alloantigens are Ly 1^{+}2^{-}3^{-} (10) whereas *H-2K* and *H-2D*-specific cytotoxic T cells (11) and specific suppressor T cells (32) are Ly 1^{-}2^{+}3^{+}. As a first step in elucidating the basis for the differential response of Ly 1^{+}2^{-}3^{-} and Ly 1^{-}2^{+}3^{+} cells to H-2 associated antigens, we have Ly-typed T cells responding in MLC to mutant H-2K and H-2D antigens, detectable in both MLC and CML. The results reported in this communication demonstrate that the B6-*Ly 1*^{a} T lymphocytes proliferating in response to B6-*H-2*^{ba} and B6-*H-2*^{bf} stimulators are Ly 1^{+}2^{+}. These observations are in contrast to those indicating that the B6-*Ly 1*^{a} responders to H-2K- and I-incompatible B10.A(1R) stimulators are Ly 1^{+}2^{-}. Based on numerous prior observations by others (12, 13) we presume that the proliferative response by B6-*Ly 1*^{a} T cells to B10.A(1R) stimulators was primarily specific for foreign I region determinants. Therefore, we have demonstrated that responder T cells specific for I-incompatible stimulators are Ly 1^{+}2^{-}3^{-}, whereas T cells required for proliferation in MLC to H-2K-incompatible stimulators are Ly 1^{+}2^{+}. While we have not yet established with certainty that the proliferating cells themselves are Ly 1^{+}2^{+}, preliminary data from experiments involving antiserum +C treatment 5 days after in vitro stimulation, but before [^{3}H]thymidine addition support the contention that the proliferating cells are Ly 1^{+}2^{+}. The obvious conclusion from these observations is that at least some of the T lymphocytes proliferating in response to foreign alloantigeneic determinants in primary MLC need not be limited to the Ly 1^{+}2^{-} subpopulation. It would appear that the subpopulation of responsive T cells is determined by either the identity of the foreign alloantigenic determinants or the molecule carrying those determinants. To resolve this question we have initiated studies to Ly type T cells proliferating in MLC to alloantigens defined by combinations of responders and stimulators differing at only the *H-2K* or *H-2D* regions.

This is not the only system in which Ly 1^+2^+ cells have been implicated. Shiku et al. reported that cytotoxic effector cells for syngeneic tumors were Ly 1^+2^+ (33). These results have been recently expanded by Stutman et al. (34) who showed that the effectors were actually Ly 1^-2^+, but that an Ly 1^+2^+ amplifier was required for efficient expression of that cytotoxicity. Similarly Cantor and Boyse reported that an Ly 1^+2^+ population was required for the generation of killer cells specific for TNP modified syngeneic cells but that the killers themselves were Ly 1^-2^+ (35). Earlier Pang et al. showed that depletion by either anti-Ly 1^+ or anti-Ly 2^+ would inhibit a secondary, virus-specific cytotoxic response but they did not perform the critical reconstitution experiment necessary to determine if an Ly 1^+2^+ cell was required (36). In contrast to the data here for H-2 mutant products Pang et al. could demonstrate no effect of anti-Ly 2 on the secondary virus induced proliferative response. We should point out that the mutant stimulated MLC is the incorporation equivalent to that seen in most *I* region differences. Thus this makes the observation reported here that a Ly 1^+2^+ is required for proliferation stimulated *K* or *D* mutants even more striking.

The observations reported in this communication are important for the understanding of the development and maturation of T lymphocytes. Previous hypotheses of T cell differentiation have proposed that Ly 1^+2^+ cells serve as progenitors for more mature Ly 1^+2^- and Ly 1^-2^+ T cells (10). Our results in MLC reject this simple progression as an all inclusive explanation for T cell differentiation. T cells capable of responding immunologically to in vitro stimulation in MLC must exist within the Ly 1^+2^+ subpopulation of a normal animal. Before a clear impression of T lymphocyte development and maturation can be obtained, a more extensive characterization of the Ly-differentiation antigen phenotype of virgin and mature T cells is required.

Summary

We have determined the Ly phenotype of the T lymphocytes which proliferate in response to mutant H-2K and H-2D alloantigens in primary mixed lymphocyte culture. Responder T cells proliferating in reciprocal cultures of H-2^d(K^dD^d) and H-2^{da}(K^dD^{da}) lymphocytes were typed Ly 2^+ through selective depletion with specific alloantiserum plus complement. Further, B6-Ly 1^a lymphocytes proliferating in response to B6-H-2^{ba} and B6-H-2^{bf} stimulators were typed as Ly 1^+2^+ through similar analysis. These results are discussed with regard to their impact on views of lymphocyte differentiation and factors determining the identity of alloreactive lymphocytes.

We thank Glen Matsushima for his help in antiserum production.

Received for publication 16 December 1977.

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MORPHOLOGICAL AND HISTOCHEMICAL ANALYSES OF TWO HUMAN T-CELL SUBPOPULATIONS BEARING RECEPTORS FOR IgM OR IgG*

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Heterogeneity of distinct subpopulations of thymus-derived (T) lymphocytes in mice has been defined by assays that discriminate T-cell functions, surface alloantigens, and the presence or absence of receptors for IgG (1, 2). Recently, subpopulations of the human T lymphocytes that form rosettes with sheep erythrocytes have been defined (reviewed in 3, 4). Two functionally distinct subpopulations can be identified and isolated by their ability to bind either IgM or IgG immune complexes. So far we have shown that T cells with receptors for the Fc portion of IgM (T_M)[1] and T cells with receptors for the Fc portion of IgG (T_G) respond differently to phytohemagglutinin, react in a similar manner to concanavalin A, and exert opposing effects on B-cell responses to pokeweed mitogen (PWM) in vitro (5, 6). T_M lymphocytes provide the required help for B-cell proliferation and differentiation into plasma cells in response to PWM, whereas T_G lymphocytes do not help but rather can suppress the proliferation and differentiation induced by helper T_M cells (6).

Here we describe studies demonstrating that the two T-cell subpopulations, T_M and T_G, in human peripheral blood have distinguishing morphological and histochemical features easily visualized at either the light or electron microscope levels.

Materials and Methods

Preparation of Cell Suspensions. Lymphocyte preparations from peripheral blood samples of normal human volunteers or from normal umbilical cord blood were isolated by techniques described in detail elsewhere (6); here the basic protocol is briefly outlined. Mononuclear cells were obtained by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, N. J.) density-gradient separation of defibrinated whole blood. T lymphocytes were allowed to form rosettes

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* Supported in part by grants CA 16673, CA 13148, and 5MO1-RR32, awarded by the National Institutes of Health, and grant 1-354, awarded by The National Foundation, March of Dimes.

[1] *Abbreviations used in this paper:* ANAE, α-naphthyl acid esterase; DMSO, dimethylsulfoxide; FCS, fetal calf serum; OE-IgG, ox erythrocytes coated with rabbit IgG antibodies; OE-IgM, ox erythrocytes coated with rabbit IgM antibodies; PAS, periodic acid-Schiff; PWM, pokeweed mitogen; SE_N, neuraminidase-treated sheep erythrocytes; T_G, T cells with receptors for the Fc portion of IgG; T_M, T cells with surface receptors for the Fc portion of IgM.

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with neuraminidase-treated sheep erythrocytes (SE_N), and the rosetting T cells then separated from the other mononuclear cells by pelleting them through two Ficoll-Hypaque gradients. The B cell-enriched fraction of blood mononucleated cells was obtained as a by-product of T-cell depletion, performed twice by this technique. T_G cells were isolated from freshly prepared T cells by further density-gradient separation of the subpopulation of T cells that formed rosettes with ox erythrocytes coated with rabbit IgG antibodies (OE-IgG). After erythrocyte lysis, the T_G cells were routinely incubated overnight before further analysis. Blood T cells depleted of T_G cells were cultured overnight in TC 199 medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 20% fetal calf serum (FCS). T_M cells were isolated then from the T_G cell-depleted population of T cells by density-gradient separation of those T cells that formed rosettes with OE coated with rabbit IgM antibodies (OE-IgM).

For comparative analysis with isolated lymphoid cell subpopulations, routine smears of fresh whole blood were prepared.

Cell Cultures. Unseparated T cells and T_G cells were cultured in Falcon plastic tubes (Falcon 2001, Div. BioQuest, Oxnard, Calif.) for 48 h at a concentration of 10^6 cells/ml in RPMI 1640 (Grand Island Biological Co.) containing 20% heat-inactivated FCS, 2 mM glutamine (Grand Island Biological Co.), and 50 μg/ml gentamicin (Schering Corporation, Kenilworth, N. J.). In some experiments, cells were cultured in the presence of cytochalasin B (Calbiochem., San Diego, Calif.) dissolved in dimethylsulfoxide (DMSO) at a concentration of 50 μg/ml of medium. Control cells were cultured in medium containing the same amount of DMSO.

Light Microscope and Histochemical Analysis. Cell suspensions were sedimented onto glass slides using a cytocentrifuge (Shandon Southern Instruments Inc., Sewickley, Pa.). For morphological studies, cells were fixed in methanol for 15 min and stained with May-Grünwald-Giemsa.

For cytochemical demonstration of enzyme activities, cells were fixed after or before cytocentrifugation. The latter procedure proved to be advantageous for evaluation of α-naphthyl acid esterase (ANAE) activity. This allowed a shorter incubation period and improved the detail of histochemical localization.

For ANAE activity cell suspensions were fixed in cold Baker's formalin calcium (pH 6.7) for 10 min and cytocentrifuged. After they were washed in distilled water, the slides were incubated for 60 min at 37°C with hexazotized pararosaniline and α-naphthyl acetate at pH 5.8 (7). The positive control for this reaction was a preparation of adherent cells separated from human peripheral blood. The adherent cells were detached by lidocaine treatment before fixation and cytocentrifugation (8).

Peroxidase activity was detected after fixation for 15 min at 4°C with 2% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4. Slides were incubated for 30 min at room temperature in 3,3'-diaminobenzidine tetrahydrochloride according to the method of Graham and Karnovsky (9). Positive controls for this histochemical reaction were white blood cells adherent to glass slides, and granulocytes prepared by dextran sedimentation; they were treated in parallel with the lymphocyte suspensions.

For histochemical detection of acid phosphatase, cytocentrifuged preparations were fixed for 30 min in cold formalin-sucrose and incubated in sodium-β-glycerophosphate for 30 min at 37°C following the Gomori technique as modified by Holt and Hicks (10). Cryostat sections of rat liver and kidney fixed overnight in cold formalin-sucrose were the positive controls for this reaction.

Neutral and acidic glucosaminoglycans were localized via the periodic acid-Schiff (PAS) reaction in cells fixed in Carnoy's fluid and counterstained with Alcian blue at pH 2.5.

Assay for C2 Synthesis. Supernates for 48-h cultures of purified T_M and T_G cells were assayed for C2 activity by Dr. Harvey R. Colten, Children's Hospital, Boston, Mass., by a previously described method (11).

Immunofluorescence Staining. Viable suspensions of cells were stained with the globulin fraction of a rabbit anti-monkey thymocyte antiserum conjugated with fluorescein isothiocyanate (molar F/P ratios, 3-6) (12) or with purified goat antibodies to human $F(ab')_2$ fragments conjugated with tetramethylrhodamine isothiocyanate (P/R ratio, 1.5). After thorough washing in RPMI 1640, containing 5% FCS and 0.1% sodium azide, cells were examined with a Leitz Orthoplan microscope (E. Leitz, Inc., Rockleigh, N. J.) equipped with incidence light illumination and phase contrast optics (13).

Electron Microscope Analysis. The purified T-cell population and separated subpopulations were fixed for 30 min at 4°C in 1.25% glutaraldehyde in 0.1 phosphate buffer, pH 7.4, washed in the buffer, and postfixed for 1 h at 4°C in 1% osmium tetroxide. After fixation the cells were dehydrated and embedded in Epon 812 (Epoxy Resins, Shell Chemical Co., Houston, Texas, 14). Thin sections cut with an ultramicrotome were stained with uranyl acetate and lead citrate and examined under a Siemens Elmiskop 101 transmission electron microscope (Siemens Corp., Iselin, N. J.).

Results

Use of Discriminating Markers to Establish the T-Cell Nature of the Isolated T_M and T_G Subpopulations of Cells. The T_M and T_G subpopulations were isolated from a total T-cell fraction that formed SE_N rosettes. However, the possibility that a minor population of B cells or macrophages contaminating the SE_N-rosetted cells might be selected with the T_G fraction through binding of OE-IgG via an Fc receptor was considered.

Purified T_G cells, T_M cells, and unfractionated T cells were incubated overnight and mixed again with SE_N under the standard conditions for rosette formation. Approximately 85% of the cells in all three populations formed SE_N rosettes.

Additional evidence supported the T-cell nature of T_M and T_G lymphocytes. All of the cells in both populations were stained by the fluorescein-conjugated globulin fraction of an anti-T-cell heteroantiserum. They lacked surface immunoglobulin and complement (C3b and C3d) receptors characteristically found on B lymphocytes (6). These observations indicated that the T_G cells, as well as T_M cells and unfractionated T cells, possess a receptor for SE_N and that this receptor becomes undetectable on a small proportion of T cells after culture.

Cytoplasmic markers for cells of the granulocytic and the monocytic-macrophage series, endogenous peroxidase and acid phosphatase, were not detected in purified T_M- or T_G-cell preparations. These enzyme activities were present in granulocyte and macrophage preparations as well as in rat liver and kidney sections used as controls. Finally, C2 activity was not found in the supernates of cultured T_M and T_G cells although macrophage synthesis of C2 is easily detectable by the assay used.

Morphology of T_M and T_G Subpopulations at the Light Microscope Level. Human T lymphocytes, when isolated from the peripheral blood by rosetting twice with SE_N and stained with the May-Grünwald-Giemsa dye mixture, were morphologically heterogeneous. The majority of the T cells, 80–90% in adult blood and 60–70% in cord blood, were small (9.3 ± 1.3 μm in diameter when measured in cytocentrifuged cell samples) and exhibited typical lymphoid morphology. These cells had a large nucleus, densely stained in patches, and a thin rim of basophilic cytoplasm (Fig. 1). The remaining cells were larger (14.2 ± 1.7 μm in diameter) and displayed a homogeneously stained nucleus surrounded by abundant cytoplasm that was either neutrophilic or slightly basophilic. The latter cells were readily identifiable by the presence in the cytoplasm of azurophilic granules which varied in size, number, and distribution from cell to cell (Fig. 2). The granules were not stained by the PAS-reaction or by Alcian blue (pH 2.5), and therefore lacked constituents with the histochemical characteristics of glucosaminoglycans. Among similarly stained cells in fresh whole blood smears, cells with features identical to those described for isolated T_G cells could be identified in appropriate frequencies. This observation makes it

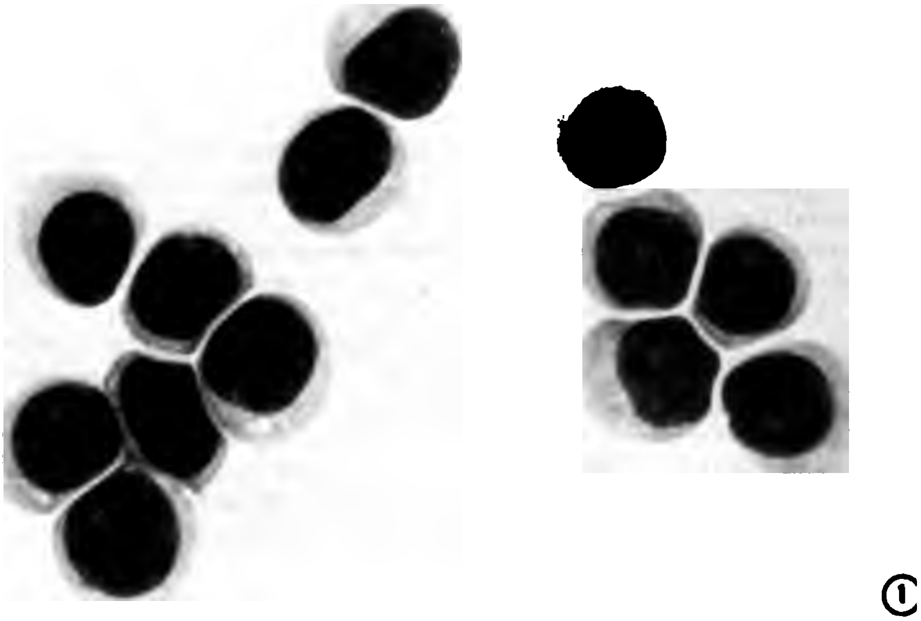

FIG. 1. T_M-lymphocyte preparation stained with May-Grünwald-Giemsa. The morphological homogeneity of this cell type is shown in two microscope fields. A large nucleus with patchy chromatin is surrounded by a thin rim of lightly basophilic cytoplasm that does not contain visible granules.

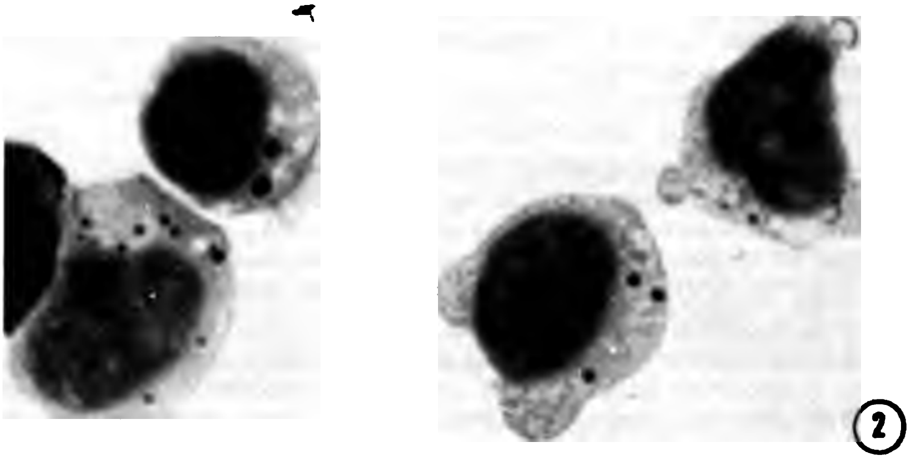

FIG. 2. T_G-lymphocyte preparation stained with May-Grünwald-Giemsa. Compared with T_M cells, T_G lymphocytes are larger, with lower nuclear to cytoplasmic ratios and a more homogeneous distribution of the nuclear chromatin. In most T_G cells, the cytoplasm contains azurophilic granules that vary in number, size, and distribution.

unlikely that the characteristic features of T_G cells described here resulted from artifactual changes during the isolation procedures.

Morphological analysis of T-cell subpopulations isolated by the presence of receptors either for IgM or IgG, revealed that all of the cells in the T_M-cell fraction had the characteristics of the type of small lymphocytes described above, whereas the T_G cells were identified as the large cells with cytoplasmic granules. In five experiments in which 400–500 cells were counted in each fraction, lymphocytes with the morphological characteristics of the T_G subpopulation were not observed among the T_M cells and vice versa.

Characteristic Localization of Esterase Activity in T_M Cells. When unseparated mononuclear blood cells, taken from the interface of a Ficoll-Hypaque density gradient, were stained for esterase activity, macrophages were intensely stained (Fig. 3A), and a large proportion of the lymphocytes contained one or two large cytoplasmic accumulations of esterase activity. 70–80% of T cells separated as SE_N rosettes were esterase-positive (Fig. 3A); a single large spot or sometimes two such vesicles were the only sites of activity detectable within the cytoplasm. Separation of T-cell subsets according to the presence of receptors for IgM or IgG showed that up to 95% of the T_M cells were esterase-positive (Fig. 3B). 90% of the T_G cells were completely esterase-negative (Fig. 3C). Less than 1% contained one or two of the large "T_M-type" accumulations of esterase activity and ~10% contained dispersed small granules with esterase activity presumed to represent lysosomes. In the B-enriched population, ~40% of the cells exhibited the latter pattern of esterase activity (Fig. 3D).

Using whole blood smears, other investigators have observed lymphocytes with cytoplasmic ANAE activity in the same large "spot" pattern that we show here to be characteristic of T_M cells (15, 16). In a direct comparison of this ANAE activity pattern among lymphocytes on whole blood smears and lymphocytes isolated from the interface of a Ficoll-Hypaque density gradient, we found the same frequencies of lymphocytes displaying the ANAE activity pattern of T_M cells in both preparations. This indicates that artifactual changes in ANAE activity during lymphocyte isolation procedures are negligible, and suggests that the T_M cells can be accurately enumerated by histochemical evaluation of ANAE activity in lymphocytes on routine blood smears.

Phagocytosis of OE-IgG by T_G Cells. A variable percentage of T_G cells in adult blood (5–10%) as well as in umbilical cord blood was observed to have phagocytosed OE-IgG within 30 min of incubation. (Phagocytosis of OE-IgM by T_M cells or of unsensitized OE by T_G cells was not observed.) Although adult T_G cells contained only one to two phagocytosed erythrocytes, some newborn T_G cells contained as many as five red cells. We noted no characteristic redistribution or loss of the azurophilic granules in T_G cells that had engulfed erythrocytes. Further studies of this phenomenon under conditions optimal for phagocytosis were not done.

Morphology of T_M and T_G Subpopulations at the Electron Microscope Level. Examination of the fine structure by transmission electron microscopy confirmed light microscope observations showing that T_M and T_G cells have different structural characteristics and that very little morphological variation exists within each population. The ultrastructural appearance of T_M cells

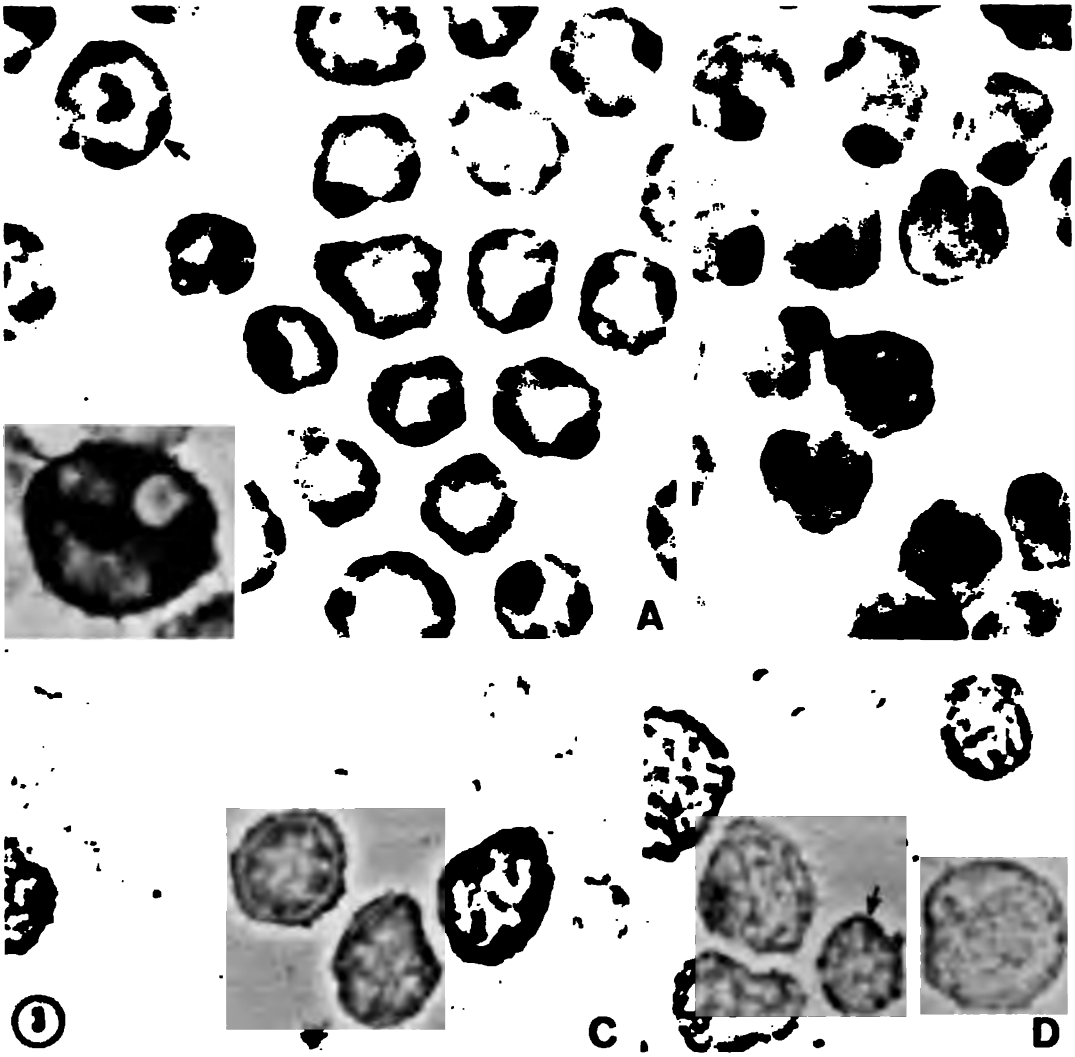

FIG. 3. Demonstration of ANAE activity in lymphocyte subpopulations. (A) Most of the SE_N-rosetted T cells show one or two large cytoplasmic spots of ANAE positivity, occasional cells have small dispersed dots of esterase activity (arrow), and some cells appear to be ANAE-negative; the *inset* shows the intense ANAE-reaction of a macrophage. (B) The great majority of T_M cells separated as rosettes with OE-IgM show striking focal accumulations of cytoplasmic ANAE activity. (C) T_G cells separated as rosettes with OE-IgG are mostly ANAE-negative; in a few of them (~10%), the histochemical reaction stains small granules dispersed within the cytoplasm. (D) B cell-enriched preparations (peripheral blood mononuclear cells twice depleted of SE_N-rosetting T lymphocytes) are either ANAE-negative or show the fine positive granular pattern (arrow) occasionally seen among T_G cells. The cells shown in (C) and (D) were photographed using phase contrast illumination, whereas those in (A) and (B) were not. × 1,250.

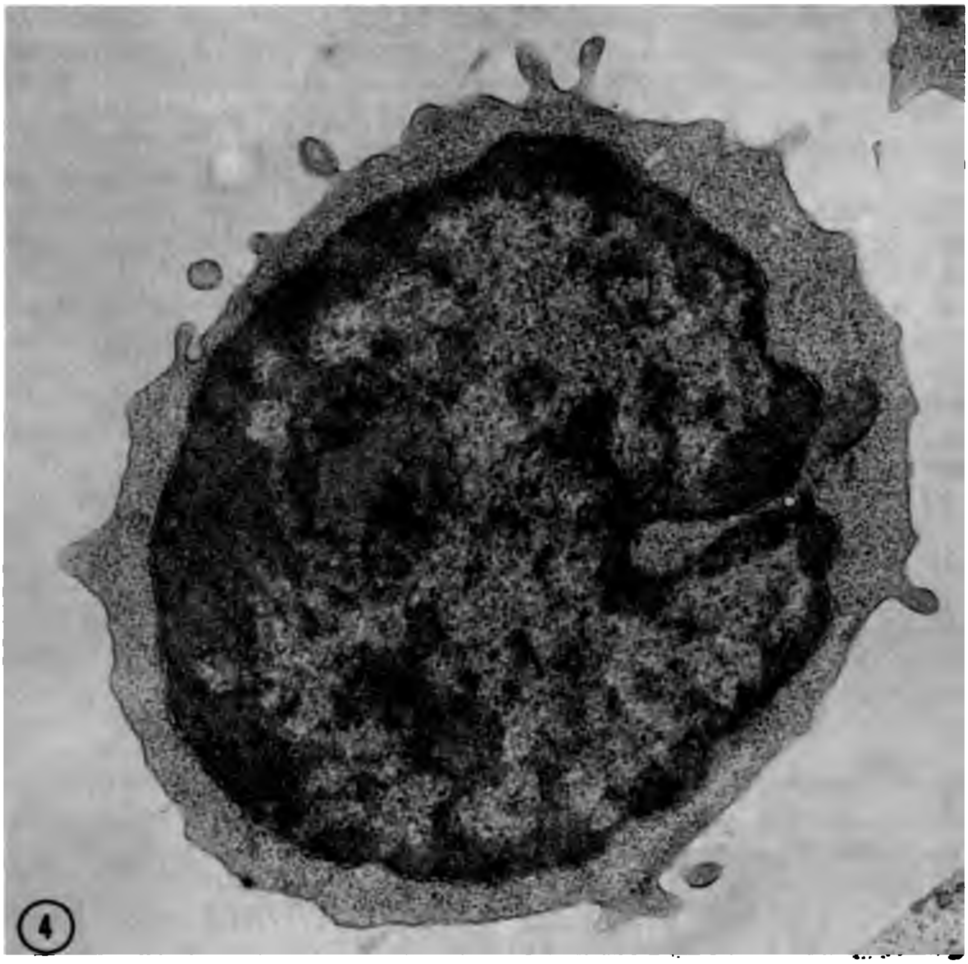

FIG. 4. Fine structural features of a T_M cell. The nucleus, with a single deep indentation, shows marginated heterochromatin and a prominent nucleolus. The cytoplasm appears devoid of the common cytoplasmic organelles, with the exception of monoribosomes. The cell surface is relatively smooth, with isolated, short microvillous projections. × 20,000.

correlated well with the general morphological features of typical small- or medium-sized lymphocytes. These cells showed a high nuclear to cytoplasmic ratio with marginated heterochromatin together with a patchy chromatin distribution in the nucleus. Other distinguishing features included a small Golgi apparatus, rare mitochondria, occasional inclusion bodies and lipid droplets, isolated profiles of rough endoplasmic reticulum, and monoribosomes uniformly dispersed throughout the hyaloplasm. An ultrastructural correlate of the large cytoplasmic accumulation of ANAE activity was not noted in the thin sections of T_M cells examined. The cell surface of T_M cells appeared smooth with only occasional short microvillous projections visible in the plasma membrane of sectioned cells (Fig. 4).

As compared with T_M cells, T_G cells showed a lower nuclear to cytoplasm ratio

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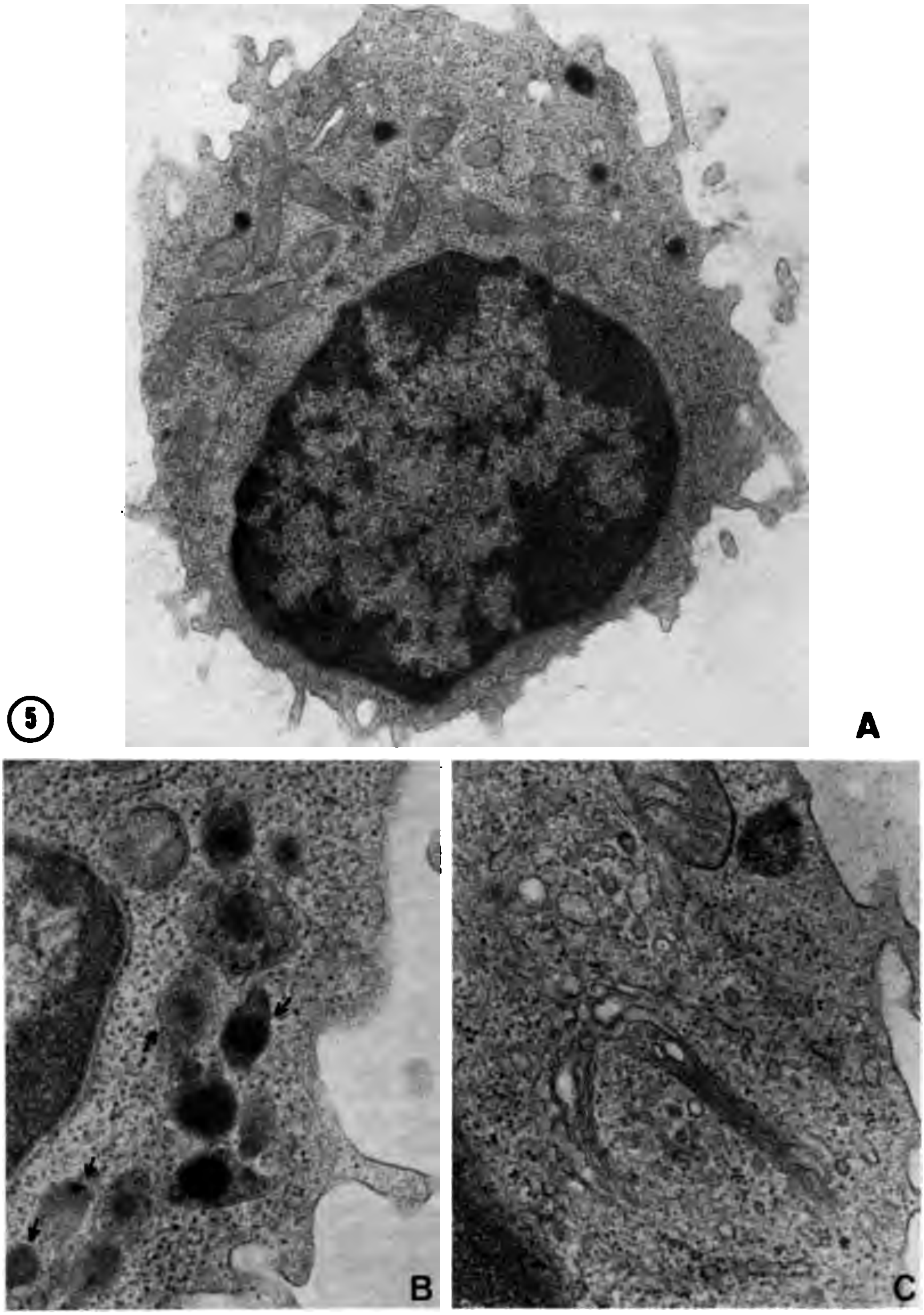

and greater margination of nuclear heterochromatin (Fig. 5 A). The Golgi apparatus was typically more extensive (Fig. 5 C) as was the rough endoplasmic reticulum; mitochondria were more numerous, often occurring in clusters. Similar density and distribution of monoribosomes were seen in T_G and in T_M cells. The T_G-cell surface appeared relatively rough as a result of the presence of numerous microvilli of variable lengths (Fig. 5 A).

The cytoplasmic granules, which were a distinguishing feature of T_G cells viewed under the light microscope, had no apparent consistent relationship with the cell organelles mentioned above, except for their frequent proximity to the Golgi apparatus. Firm morphological evidence for Golgian origin of the granules, however, was not established. Each granule was surrounded by a membrane unit and contained a matrix which was either homogeneously electron dense or contained a single darker inclusion; an electron transparent space often separated the matrix from the limiting membrane (Fig. 5 B).

Phagocytosis of OE-IgG by T_G cells was also documented at the electron microscope level (Fig. 6) after a 12-h incubation interval before cell fixation. Fusion of the cytoplasmic granules with the phagosome was never observed.

Effect of Cytochalasin B on Release of Granules from Cultured T_G Lymphocytes. The percentages of lymphocytes with T_G characteristics in the unseparated T-cell preparations, determined by counting cells with granules on slides stained with May-Grünwald-Giemsa, closely correlated with the number of cells forming OE-IgG rosettes in both adult blood (12 compared with 13.5%) and cord blood (28 vs. 33.5%). However, in five separate experiments, only 55–85% of the cells in the purified T_G-cell preparations showed detectable granules although all of these cells exhibited other morphologic features characteristic of T_G cells. This suggested the possibility of some release of granules from T_G lymphocytes during the cell separation procedures and overnight incubation before examination.

To test this hypothesis, two T_G fractions containing 55 and 81% cells with granules were cultured for 48 h. In the absence of cytochalasin B only 8 and 16% of the cells, respectively, carried detectable granules at the end of the culture period, whereas in the presence of cytochalasin B (50 μg/ml) the percentages in both cases were close to the preincubation values (51 and 72%, respectively). Cytochalasin B was therefore able to prevent loss of granules from T_G cells.

Discussion

We have previously described two functionally distinct subpopulations of human T cells based on their ability to bind the Fc portion of IgM (T_M) or IgG

FIG. 5. Fine structural features of T_G cells. (A) In comparison with T_M cells, the cytoplasm is more extensive and contains numerous mitochondria, profiles of rough endoplasmic reticulum, and Golgian cisternae. Several electron-dense granules are scattered within the cytoplasm. The cell surface is relatively rough with long and slender microvillous projections. × 14,000. (B) T_G granules are surrounded by a membrane unit and contain an electron-opaque matrix, often showing a more electron-dense core; the morphological heterogeneity of T_G granules (arrows) possibly reflects stages of granular maturation. × 40,000. (C) A higher magnification of a cytoplasmic area of a T_G cell illustrates a well-developed Golgi apparatus. × 36,000.

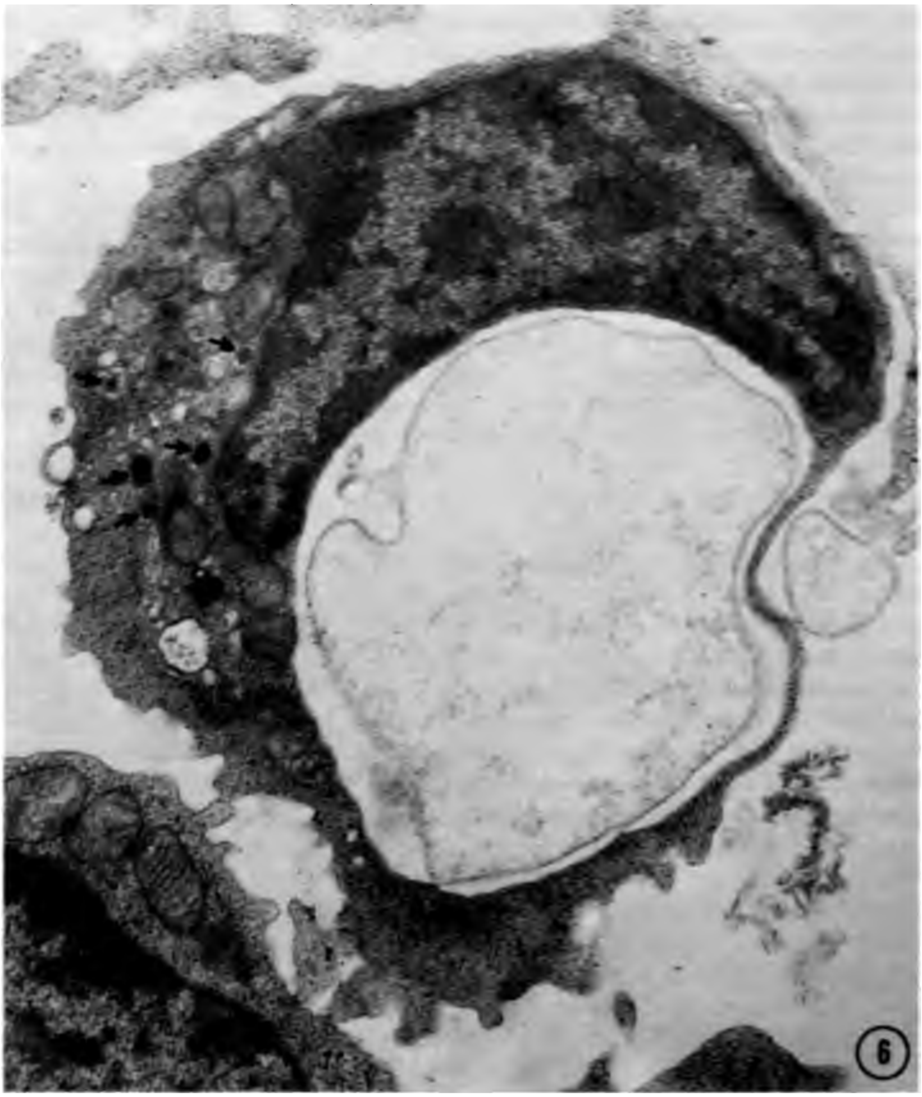

FIG. 6. Phagocytosis of OE-IgG by T_G lymphocytes. The cell contains a red cell within its phagosome. T_G granules (arrows) do not show any relationship with the phagosome. × 14,000.

(T_G) (3, 5, 6). In this study we have shown that not only do these subpopulations differ functionally but they are also easily distinguished on the basis of their morphological and histochemical characteristics.

Efficient purification of the newly described subpopulations of T lymphocytes was considered a primary requirement for these studies. It was deemed especially important to exclude that a contaminant cell belonging to another mononuclear cell line had been enriched for by the separation techniques employed. For this reason, we have examined purified T_M and T_G subpopulations extensively for markers identifying B cells, and monocytes as well as T cells.

The results reported here and in a previous communication (6) indicate that both subpopulations are T lymphocytes and are not significantly contaminated with other mononuclear cell types. The apparent morphological homogeneity of the purified fractions as visualized at both light and electron microscope levels substantiates the latter conclusion.

A number of general structural features distinguish T_M and T_G cells, including differences in cell size, nuclear to cytoplasmic ratio, and the extent of development of cellular organelles. On the basis of our observations, we cannot exclude the possibility that some of these morphological differences (e.g., the difference in the villous projections from the surfaces of T_M and T_G cells) could be due to changes induced by cell activation during the isolation procedures. Certain characteristic features of T_M and T_G cells, however, were clearly seen in unseparated whole blood preparations, thus indicating that these differences were not a result of cell modification induced during their separation. One striking characteristic of T_M cells was their distinct cytoplasmic accumulations of ANAE activity; the large spots of ANAE staining, not found in T_G and B cells, were also very different from the ANAE reaction pattern of macrophages, and thus may serve as a practical marker for rapid enumeration of cells of this subpopulation.

T_G cells, on the other hand, contained characteristic granules that could be visualized under both light and electron microscopy. These granules did not contain the enzyme markers of lysosomes in granulocytes and macrophages, nor were they ever observed in confluence with phagosomes containing OE-IgG. Within individual T_G cells, we noted considerable heterogeneity in the morphological appearance of the single membrane-lined granules. They differed in size, electron density of the matrix, and presence or absence of a central dense core. This type of heterogeneity is suggestive of a maturational pattern as defined in other cell types, e.g., the protein-secreting serous cell (17, 18). The release of the granular contents from T_G cells with suppressor activity (6) after in vitro incubation at 37°C and the inhibition of this release by cytochalasin B, a drug known to affect microfilaments, raise the possibility that the granules contain suppressor substances. Support for this hypothesis must await further studies on the mechanism of suppression by T_G cells and on the nature and function of the material enclosed within the granules. For the present, these characteristic granules may serve as a convenient additional marker for T_G cells in certain lymphoproliferative and immunodeficiency diseases.

Endocytosis of OE-IgG by a significant proportion of the lymphocytes in purified T_G-cell preparations was an unexpected finding that seems especially noteworthy. The possibility that such cells represented a contaminating population of non-T phagocytic cells would appear to be excluded by the fact that they lacked the markers characteristic of cells of the monocyte-macrophage and granulocytic series. Moreover, these erythrophagocytic cells were indistinguishable from other T_G cells on the basis of their morphological, histochemical, surface antigen, and sheep erythrocyte-binding characteristics. In view of the possession of receptors for the Fc portion of IgG by T_G cells, perhaps this ability to engulf erythrocytes coated with IgG is not surprising. Nevertheless, comparison of the mechanisms by which T_G cells and classic phagocytic cells accomplish this feat, and elucidation of the fate of the endocytosed cells, could shed new light on the biologic functions of cells within the T_G subpopulation.

Summary

Two subpopulations of circulating human T cells forming rosettes with neuraminidase-treated sheep erythrocytes were purified on the basis of the presence of receptors for IgG (T_G cells) or for IgM (T_M cells), and were shown to have distinguishing morphological and histochemical characteristics. T_M cells had the general features of typical small- or medium-sized lymphocytes; most were easily identifiable by distinctive cytoplasmic accumulations, usually one and sometimes two large spots, of nonspecific acid esterase activity. The larger T_G cells had a more complex system of cytoplasmic organelles, numerous surface villous projections, and distinctive vesicles in their cytoplasm. These vesicles were lined by a unit membrane enclosing granular material of varied electron density and intravesicular distribution. The release of the vesicular contents on short-term culture of T_G cells was inhibited by cytochalasin B. Definition of these distinguishing characteristics of T_M and T_G cells provides a basis for practical enumeration of these functionally distinct subpopulations of human T cells. Some of the T_G cells were capable of endocytosis of IgG antibody-coated erythrocytes.

We wish to thank Dr. Harvey Colten for performing the C2 assay, Mrs. Diann Gurley for preparing the manuscript, and our colleagues for helpful criticisms.

Received for publication 30 January 1978.

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CELL SURFACE GLYCOPROTEINS OF MURINE CYTOTOXIC T LYMPHOCYTES

I. T 145, A New Cell Surface Glycoprotein Selectively Expressed on Ly $1^{-}2^{+}$ Cytotoxic T Lymphocytes*

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The immune system is endowed with great flexibility. It has at its disposal a large number of alternative pathways of reaction against foreign, immunogenic substances. A sizeable part of this impressive flexibility is due to the fact that a number of subsets of specialized cell types coexist and interact within this system. Among these cell types, lymphocytes have been shown to play a dominating role in both the specific induction of the immune response and in its further developments.

Within the two major groups of lymphocytes (T and B cells) there exist additional subgroups with unique functional properties. Our knowledge of T-lymphocyte subgroups stems largely from experiments using a combination of serological and functional markers to define these cells (1-4). In principle, virgin lymphocytes, although preprogrammed as to their eventual subgroup (1), normally fail to express the functional properties displayed by activated cells arising from these subgroups (5, 6). The expression of these functions associated with the activated state can be interpreted to reflect qualitative as well as quantitative differences in the surface membrane composition of the cells.

Thus, the cell surface membrane characteristics of lymphocytes are decisive factors in determining the potential activity of these cells, and although progress in this field is rapid, we still have a largely imperfect understanding of how these membrane components effect and regulate immune reactivity.

In this article we have focused our attention on T lymphocytes and their membrane glycoprotein composition after induction by immunogen or polyclonal T-cell activators. With the aid of fractionation procedures now available to enrich for murine T cells with a defined surface structure or function, in combination with procedures for selective labeling of cell surface glycoproteins, comparisons between virgin and immune T cells at different stages of differentiation have been made. As will be demonstrated, this approach has been a highly fruitful one, allowing the successful characterization of a new T-lymphocyte unique surface glycoprotein associated with a defined subgroup of cells and expressed in conjunction with the cytolytic stages of T-cell reactivity. The implications of these findings will be discussed.

Materials and Methods

Mice. All strains of inbred mice reported in this article have been raised and maintained in our breeding facilities in the Department of Immunology, University of Uppsala. Both male and female mice 6-16 wk of age were used in these studies.

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* Supported in part by the Swedish Cancer Society, and National Institutes of Health grant AI 13485-02.

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Conditions for the Generation and Purification of Cytotoxic T Lymphocytes

ALLOGENEIC SENSITIZATION: In vitro and in vivo conditions for the allogeneic sensitization of purified splenic T cells have been described in detail elsewhere (6). In brief, spleen T cells purified by passage through Ig-anti-Ig glass bead-coated columns (7) were responded in culture against 2,000 R irradiated allogeneic spleen cells, or injected intravenously into lethally irradiated (800 R) allogeneic hosts. In both systems of sensitization, responding cells were collected at the peak of the cytotoxic response (day 5–6).

CONCANAVALIN A ACTIVATION: Concanavalin A (Con A)[1] blasts were prepared by incubating Ig-anti-Ig column-purified spleen T cells with a predetermined optimal concentration of mitogen (2 μg/ml). The cells were adjusted to a concentration of 10^6/ml in Eagle's-Hanks' amino acid-supplemented tissue culture media plus 5% fetal calf serum (FCS) and placed in culture flasks (3024; Falcon Plastics, Div. of Bio Quest, Oxnard, Calif.). These flasks had been incubated overnight at 37°C with syngeneic spleen cells, and washed three times with culture media to obtain the adherent cell population necessary for optimal mitogen stimulation of these column-passed cells.

Isolation of the Activated Blast Cells. The various allo- and mitogen-activated blasts were isolated from the small lymphocyte fraction and cell debris by velocity sedimentation at unit gravity through a linear 15–30% FCS gradient (8). The blast cell fractions were collected, washed three times in phosphate-buffered saline (PBS) and divided for use in cell-mediated cytotoxic reactions and for cell surface labeling.

Cell-Mediated Cytotoxicity Testing. Alloimmune T-cell cytotoxicity was assayed in v-bottom Cooke microtitre plates (220 M-25 AR; Cooke Laboratory Products, Div. Dynatech Laboratories, Inc., Alexandria, Va.) at various effector:target cell ratios using a constant number (10^4) of an in vitro maintained line of the DBA/2 mastocytoma P815. The assays were performed in a total reaction volume of 0.1 ml of RPMI-1640 media supplemented with 5% heat-inactivated FCS. 2–3 × 10^6 target cells were labeled with 100 μCi of $Na^{51}CrO_4$ (Amersham/Searle Corp., Arlington Heights, Ill. sp act 366 mCi/mg) for 30–45 min at 37°C in a total volume of 100 μl RPMI-1640 plus 5% FCS. Controlled initiation of the cytotoxic response was achieved by centrifugation of the plates at 300 *g* for 4 min at room temperature. Assays were carried out for 2 and 4 h at 37°C in a 7.5% CO_2/air atmosphere. Reactions were stopped by the addition of 0.1 ml of ice-cold PBS, followed by centrifugation in the cold (4°C). 0.1-ml aliquots of the supernates were carefully removed and counted in a gamma scintillation spectrometer. Percent specific lysis was calculated according to the method of Brunner et al. (9).

Lectin-mediated T-cell cytotoxicity by Con A-generated blasts was also assayed in v-bottom plates against ^{51}Cr-labeled P815 as described above. The total cytotoxic potential of the Con A-activated blasts was determined according to Bevan and Cohn (10) by the addition of 0.5 μg of Leukoagglutinin (Pharmacia Fine Chemicals, Uppsala, Sweden) per well and compared with identical determinations receiving the same volume of media without lectin. Spontaneous release was determined without effector cells, with and without lectin, and did not vary significantly in any case. Total isotope release was determined by sonication of the target cells at 50 Hz for 1 min. Percent cytotoxicity was calculated as above.

Enzyme-Catalyzed Cell-Surface Labeling with NaB^3H_4. The galactose-oxidase-tritiated sodium borohydride technique of Gahmberg et al. (11) has been used for the selective radiolabeling of cell-surface glycoproteins of the various cell preparations. Briefly, this labeling procedure involves the enzyme-catalyzed oxidation of exposed terminal galactosyl and *N*-acetyl galactosaminyl residues by galactose oxidase to the corresponding C6 aldehyde, which is then reduced with tritiated sodium borohydride of high specific activity. The procedure for labeling is essentially performed according to Gahmberg (11) with a few modifications to enable the labeling of small quantities of cells. Cells to be used for surface labeling were washed twice in PBS and adjusted to a concentration of 1.5 × 10^7 cell/ml in RPMI-1640 media (pH 7.4) supplemented with protease-free preparations of neuraminidase (12.5 U/ml, *Vibrio cholerae*; Behringwerke AG, Marburg, W. Germany) and galactose oxidase (5 U/ml, Kabi AB, Sweden), and were incubated in a 37°C water bath for 40 min. At the end of this period, the cells were washed twice in PBS (made pH 8.0 with 1 N NaOH), resuspended to the original cell concentration in PBS (pH 8) containing 1 mCi/ml NaB^{3}H$_4$ (Amersham-Searle; sp act 8–12 Ci/mM) and incubated at room temperature in a fume hood for 40 min. The labeling was stopped by the addition of ice-cold PBS (pH 8), followed by three washes in the cold. In cases where as few as 2 × 10^5 cells were labeled, normal syngeneic spleen or tumor cells were added with the cold PBS used to stop the reaction. The addition of cells at this point did not introduce artifactual labeling and helped considerably as carrier cells to prevent losses during the subsequent washings.

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[1] *Abbreviations used in this paper*: Con A, concanavalin A; FCS, fetal calf serum; LPS, lipopolysaccharide; MLC, mixed leukocyte culture; Mls, M-locus; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; SD, "classical" serologically defined regions of the H-2 complex (H-2K and H-2D); SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

Solubilization of NaB^{3}H$_4$-Labeled Cells. NaB^{3}H$_4$ labeled cells were solubilized at a concentration of 5 × 10^7 cells/ml. The cell pellet from the final wash after NaB^{3}H$_4$ labeling was first gently resuspended by the addition of one-half the final volume of ice-cold PBS, followed by an equal volume of ice-cold solubilizer (1% Nonidet P-40, 2 mM phenylmethylsulfonylfluoride, 10 μg/ml soy bean trypsin inhibitor, 2% vol/vol of a saturated solution of epsilon-amino caproic acid, and 10 μg/ml *N-α-p*-tosyl-L-lysine chloromethyl ketone HCl) in PBS. The cells were incubated for 20 min on ice and then centrifuged at 20,400 *g* for 20 min at 4°C. The membrane-rich supernate routinely contained 74–86% of the total radioactivity which was trichloroacetic acid (TCA) precipitable and ether-insoluble. Samples were immediately mixed with an equal volume of sample buffer containing 4% sodium dodecyl sulfate (SDS) and 0.3 M 2-mercaptoethanol (12), and then heated in a boiling water bath for 1.5 min. Samples were stored frozen (−70°C).

Polyacrylamide Slab Gels and Radiolabeled Marker Proteins. Solubilized NaB^{3}H$_4$-labeled cell preparations and marker proteins were prepared with and without reduction (12), and run in linear 7.5–10% glycerol-stabilized gradient gels containing 0.1% SDS. The gel system used was the discontinuous buffer system described by Laemmli (12).

Tritiated IgM was prepared by immune precipitation of [^{3}H]leucine-labeled supernates from lipopolysaccharide (LPS)-stimulated blasts according to the sandwich technique of Melchers and Andersson (13). DEAE-CMC 52-purified IgG$_1$ from the BALB/c myeloma MOPC 21, twice-recrystalized ovalbumin (Worthington Biochemical Corp., Freehold, N. J.), human serum albumin (Kabi AB), and purified hexon from type 2 adenovirus were labeled with carrier-free ^{125}I-Na by the chloramine T method (14) and served as marker proteins for apparent molecular weight determinations for each gel.

Fluorography. Slab gels were fixed overnight in an acetic acid-isopropanol-water mixture (1.2:3.1:8), and processed for fluorographic-autoradiography using the highly sensitive method of Bonner and Laskey (15), which enables detection of ^{3}H by the incorporation of the fluor 2,5-diphenyloxazole (PPO) into the fixed gel. Slab gels were dried by heating and vacuum suction, overlayered with RP X-Omat x-ray film (XR-2; Eastman Kodak Co., Rochester, N. Y.), wrapped in aluminum foil and exposed for a period of 1–5 days. In some cases, the x-ray film was hypersensitized with a flash of light (16) before contact with the dried gel to ensure proportionality of image darkening such that 30 cpm in a single band could be visibly detected with 72 h of exposure. Quantitative measurements of the individual gel profiles were determined on a Joyce-Loeb recording densitometer.

Results

The Appearance of a New, Distinct Surface Glycoprotein (T 145) on T Lymphoblasts Activated Across H-2 Barriers. For our analysis of distinguishing surface markers between normal and immune T cells, populations of cytotoxic T lymphocytes were generated by both primary mixed leukocyte culture (MLC) activation across H-2 barriers and by in vivo sensitization in lethally irradiated allogeneic recipients. The blast cell fractions from each of these immune cell preparations were then purified from the small lymphocyte fraction by 1 *g* velocity sedimentation and then surface-labeled with NaB^{3}H$_4$. After solubilization of the labeled cells, the glycoprotein patterns were analyzed by SDS-polyacrylamide gel electrophoresis and visualized by fluorographic-autoradiography. Nonimmune cells labeled in a similar manner included thymocytes and normal spleen T lymphocytes. Fig. 1 shows the cell surface

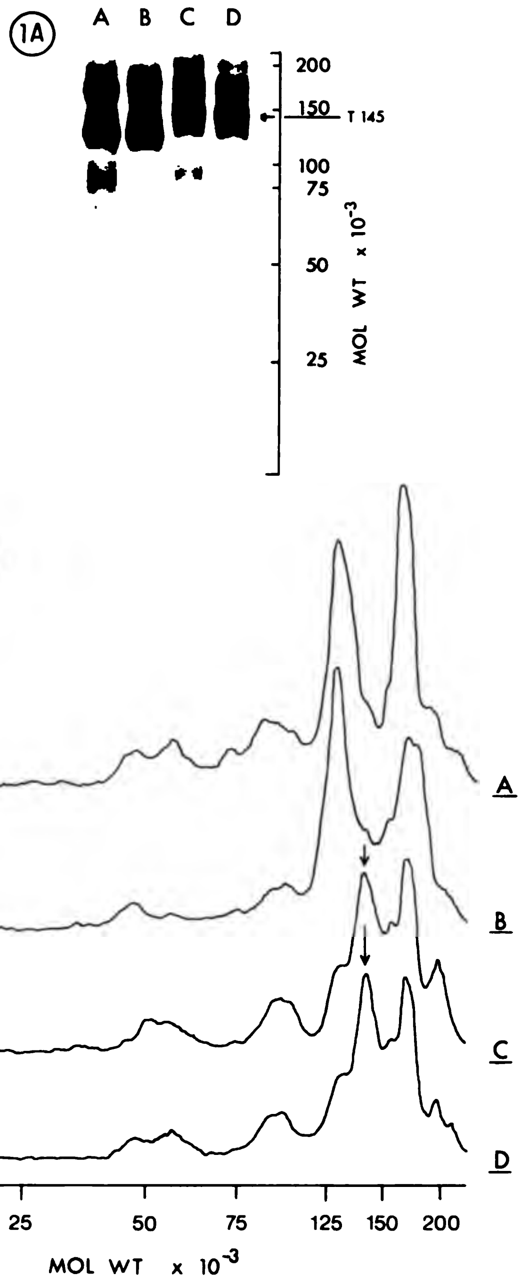

FIG. 1. Cell surface glycoprotein patterns obtained after electrophoresis of NaB^3H_4-labeled normal and immune T-cell preparations. Fig. 1 A illustrates the autoradiographic glycoprotein patterns of (A) CBA/J Ig-anti-Ig column-purified thymocytes, (B) CBA/J Ig-anti-Ig column-purified spleen T cells, (C) MLC-generated CBA/J anti-DBA/2 T blasts, (D) in vivo generated CBA/J anti-DBA/2 T blasts. The quantitative relationship of the various glycoprotein bands is seen in the densitometric tracings of Fig. 1 B. Arrows indicate the position of T 145. Each preparation, corresponding to between 5 and 10 μg of protein from the surface-labeled cell lysates were adjusted to 15,000 cpm in sample buffer (12). Electrophoresis was performed in a linear 7.5–10% gradient of acrylamide containing 0.1% SDS.

glycoprotein patterns obtained after NaB^3H_4 labeling of these normal and alloactivated T lymphocytes. Even though several glycoprotein bands appear identical between the various T-cell preparations, a number of differences are apparent. Such differences between resting and activated T cells have been previously noted by Gahmberg et al. (11). Specifically, these changes in gel patterns are seen in the appearance of large molecular weight glycoproteins at 200,000 and 145,000 daltons on activated T cells, and loss of two glycoproteins at 180,000 and 70,000 daltons. The major glycoprotein band at 130,000 daltons, which is heavily labeled on both thymocytes and peripheral T cells, can be seen here to be considerably reduced in intensity on both groups of activated T cells. As will be seen in subsequent figures, the intensity of this glycoprotein band is variable and usually less striking on activated T cells than on resting populations of T cells. This 130,000-dalton glycoprotein can, however, be clearly distinguished from the 145,000-dalton glycoprotein in that the former is apparent only after neuraminidase treatment of the cells and displays selective affinity for the T-cell lectin Helix Pomatia.[2] Furthermore, selective removal of this heavily labeled glycoprotein on Helix Pomatia affinity columns more clearly reveals the absence of the 145,000-dalton glycoprotein on resting T lymphocytes (data not shown).

In considering the significance of these changes in surface glycoprotein labeling patterns, one must take into account the obvious fact that the activated T cells are phenotypically blast-like in morphology, whereas resting T lymphocytes are characteristically small. Thus, the glycoprotein differences strictly related to immune activation cannot be distinguished from blast cell-associated glycoproteins. To discriminate between these possibilities we have examined the surface-glycoprotein patterns of a total of 15 different mouse T lymphomas as a representative panel of T-cell blasts functionally deficient in the effector cell activities associated with MLC-activated lymphoblasts (data not shown). In each case, the 200,000-dalton glycoprotein was a consistent feature of these cells with variable expression of labeled glycoproteins at 70,000 and 180,000 daltons, whereas none of these T lymphomas expressed the 145,000-dalton band. Thus, the only consistent cell-surface glycoprotein to display characteristics as perhaps related to immune activation of T lymphocytes has been the 145,000-dalton glycoprotein. As seen in Fig. 1, this glycoprotein is expressed with similar intensity on T lymphoblasts, regardless of whether sensitization was performed in vivo or in vitro. We call this distinguishing T lymphoblast glycoprotein T 145.

Further studies on the cellular distribution pattern of T 145 expression have shown normal spleen B cells, LPS blasts, and a number of B-cell lymphomas to all be T 145-negative. These results further emphasized the restricted expression of T 145 as a T-lymphoblast unique membrane glycoprotein and clearly not as a characteristic of any rapidly dividing T- or B-cell blast.

Different Alloantigenic Barriers Vary in their Ability to Induce T 145 on the Responding T Lymphoblasts. Gene products of the various regions of the H-2

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[2] B. Axelsson, A. Kimura, S. Hammarström, H. Wigzell, K. Nilsson, and H. Mellstedt. Manuscript in preparation.

TABLE I

The Relative Efficiency of Various Alloantigenic Barriers to Induce T 145⁺ Lymphoblasts

Strain combinations analyzed*	Genetic difference	Relative labeling intensity of T 145‡
B10.G → B10.T(6R)	H-2D	++++
B10.S(7R) → B10.S	H-2D	+++
CBA/H-H-2^{ka} → CBA/H	H-2K point mutation	++++
CBA/J → C57BL/6	H-2, non-H-2	++
CBA/J → DBA/2	H-2, non-H-2	+++
C57BL/6 → DBA/2	H-2, non-H-2	+++
B10.BR → B10.D2	H-2	+++
B10.BR → B10.M	H-2	++
A.TL → B10.HTT	IA, IB, non-H-2	++
B10.S(7R) → B10.HTT	IC, H-2S, H-2G	++
B10.BR → CBA/J	non-H-2, Mls	(±)
CBA/H → CBA/J	non-H-2, Mls	+
BALB/C → DBA/2	non-H-2, Mls	+

* Ig-anti-Ig column-purified T lymphocytes were reacted in MLC against 2,000 R irradiated spleen cells of the stimulating cell genotype. Blasts were isolated on day 5 of culture by velocity sedimentation at unit gravity through a linear 15–30% FCS gradient.

‡ Purified T blasts were surface-labeled with NaB^3H_4 under highly standardized conditions (see Materials and Methods) with the same batch of reagents, solubilized, and adjusted to the same total TCA-precipitable, ether-insoluble cpm. The glycoproteins of the various preparations were separated under reducing conditions by electrophoresis through a 7.5–10% gradient of acrylamide containing 0.1% SDS, fixed and processed for fluorography. The relative labeling intensity of T 145 was judged as the amount of radioactivity associated with the T 145 glycoprotein band relative to the total surface label of the individual preparations.

complex and other strong stimulating loci (Mls), have been claimed to have a differential impact as stimulating structures for various subsets of T lymphocytes (1). Using a variety of strain combinations in one-way MLCs, we have been able to select for proliferative responses against a combination of stimulating loci as well as selected regions of the H-2 complex in order to assess whether or not a specific genetic incompatibility is needed for the expression of T 145 on the responding blasts. Table I shows the relative ranking order of efficiency for the induction of T 145 expression, depending on the nature of the genetic incompatibility stimulating the MLC reaction. The most efficient incompatibility for the expression of T 145 on the responding blasts was a selective difference across the classical H-2 regions H-2K or H-2D (SD) only, with differences across the entire H-2 complex being second. T cells responding against I-region differences only do express the T 145 band, although in a lower intensity on a population basis than do the anti-SD activated T cells. MLC blasts generated in H-2 identical, Mls incompatible combinations have been the most variable in expressing the T 145 marker, and represent by far the weakest of the T blasts analyzed for the expression of T 145. As the proliferative ability of the T blasts generated against Mls and/or Ia are at least of orders of magnitude similar to

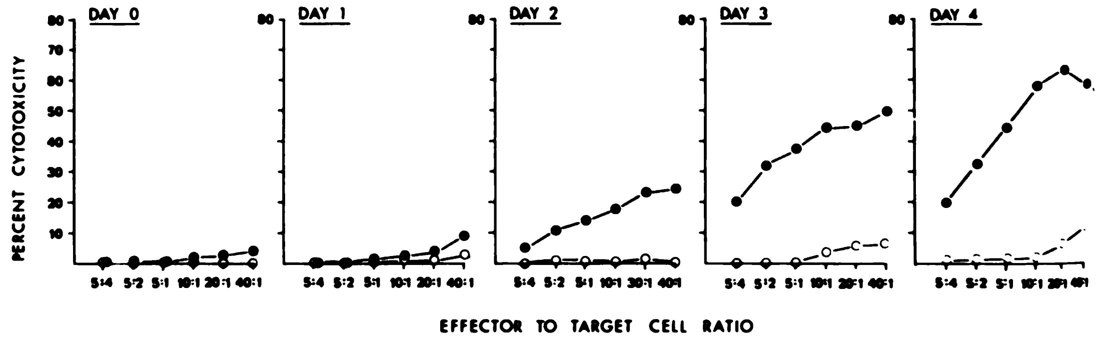

FIG. 2. Kinetics for the development of cytotoxic function by Con A-induced lymphoblasts. Cells from flask cultures were harvested at the designated times after the addition of Con A, and they were tested for cytotoxic activity in a 4-h assay against ^{51}Cr-labeled P815 tumor target cells at the indicated effector:target cell ratios. (○), cytotoxicity expressed in the absence of agglutinant; (●), total cytotoxic potential of the blast cells in the presence of 5 μg/ml of leukoagglutinin.

those against SD differences, it would thus seem clear that the T 145 glycoprotein is not merely a marker of any proliferating T lymphoblast.

Positive Correlation in Time Between the Appearance of Cytolytic Ability and Expression of T 145 on Con A-Induced Lymphoblasts. Induction of T-cell proliferation by alloantigenic differences is a nonrandom event, as different subsets of T lymphocytes appear to respond preferentially towards the various histocompatibility antigens. Thus, it is now well recognized that the cells responding towards SD differences are to a large extent Ly $1^-2^+3^+$, and express efficient cytolytic ability (1), whereas T cells reacting against Ia or Mls incompatibilities are dominated by Ly $1^+2^-3^-$ cells with less impressive killing ability (1, 18, 19). The results of the preceding section would be in line with the possibility that the T 145 band may represent a differentiation antigen typical of a cell with the functions and surface markers of cytolytic nature. Using Con A as a polyclonal activator of T cells, it would then be possible to generate T blasts with a wide range of immunological activities (5), and to follow the glycoprotein profiles of the blast cells while focusing upon only one of these activities; the appearance of cytolytic function.

To obtain optimal mitogenic stimulation and expansion of the Con A-reactive cells, Ig-anti-Ig column-purified spleen T cells from CBA/H mice (10^6/ml) were cultured with 2 μg/ml of Con A. The culture vessels were Falcon flasks preincubated overnight with syngeneic spleen cells and subsequently washed so that they contained only the adherent, catalyzing cell population. At the end of the 1st and 3rd day of culture, lymphocytes were diluted to a cell concentration of 3.5×10^5 cells/ml with fresh media containing Con A (2 μg/ml) to minimize cell death and allow optimal conditions for cell growth as previously described (20). At 24-h intervals cells were harvested from culture, adjusted to the appropriate cell concentration, and tested for cytolytic activity against allogeneic, ^{51}Cr-labeled target cells using phytohemagglutinin (PHA) as an agglutinant to provide efficient contact between effector and target cells (10).

Fig. 2 illustrates the kinetics and extent of cytolytic activity displayed by Con A-induced T lymphoblasts. As is seen, only minor cytolytic activity was detected

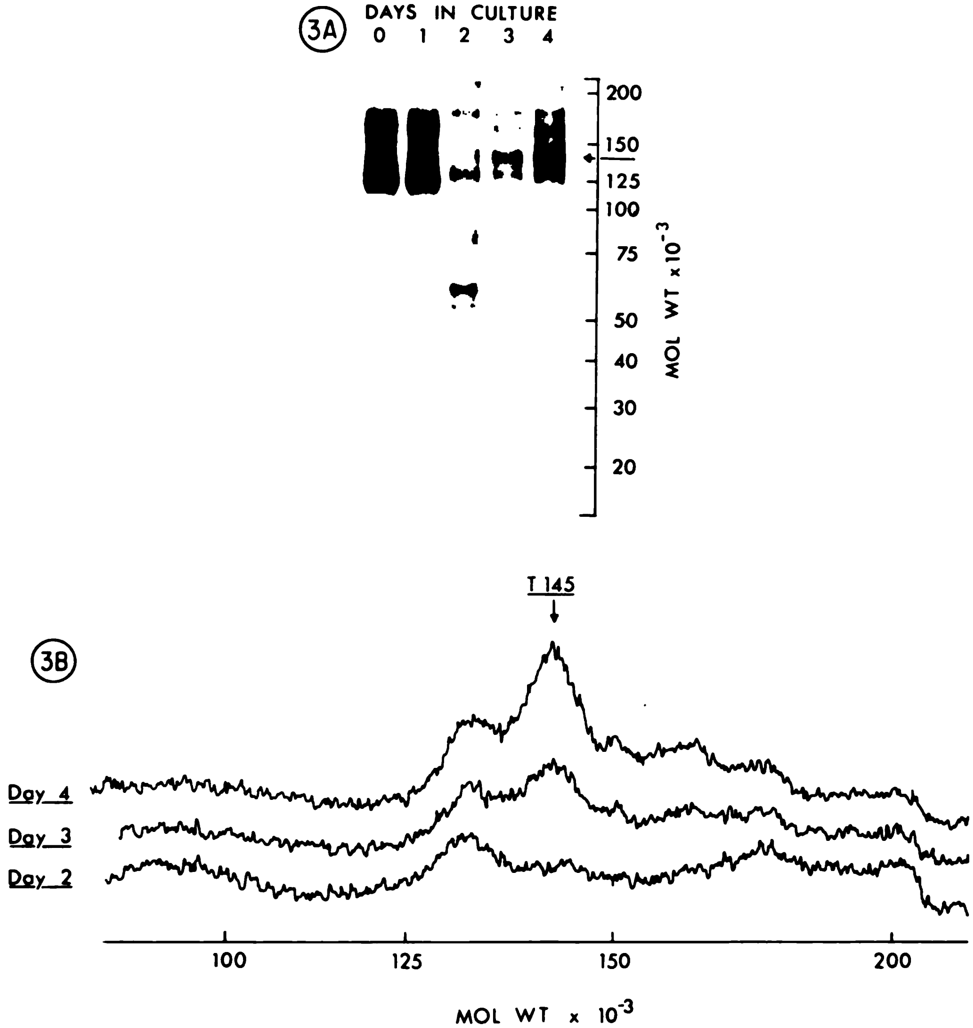

FIG. 3. Cell surface glycoprotein patterns of NaB^{3}H$_4$-labeled blasts at various times during Con A activation. Aliquots of the same Con A cell preparations assayed for cytotoxic function in Fig. 2 were washed three times in 0.05 M α-methyl-D-mannoside, surface-labeled with NaB^{3}H$_4$, solubilized, and examined for the expression of T 145 at various times after the initiation of the cultures. Fig. 3 A illustrates the fluorographic patterns of cell surface glycoproteins at the indicated days in culture. Quantitative differences in the expression of T 145 are seen in the densitometric tracings in Fig. 3 B. Each sample was adjusted to 12,500 cpm in electrophoresis sample buffer (5–10 μg protein) and separated in a linear 7.5–10% gradient of acrylamide containing 0.1% SDS.

on day 2, despite the fact that 96% of the viable cells were large blasts at this time. On days 3 and 4, the blasts were morphologically indistinguishable from those seen on day 2, and yet the killing ability of these Con A blasts increased in a drastic manner. The results of experiments carried out in parallel on the cell-surface glycoprotein patterns during Con A activation are shown in Fig. 3. As is shown, T 145 was virtually absent on days 1 and 2, and it first appeared in detectable quantities on day 3, increasing in intensity during the next day of

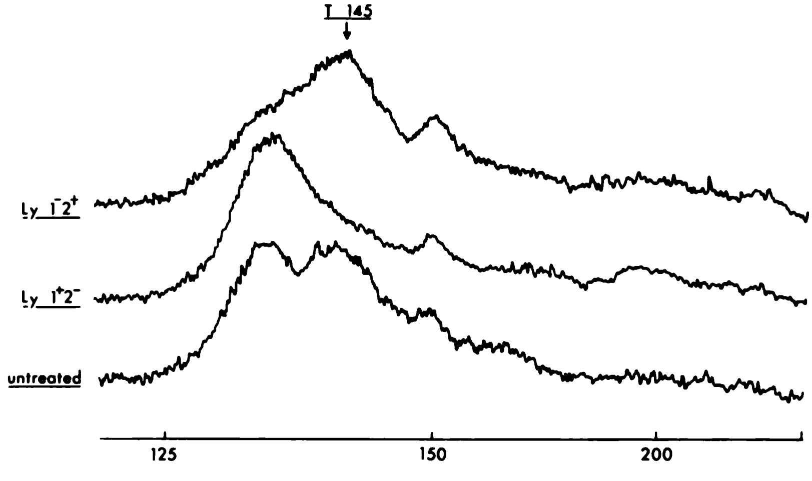

FIG. 4. Densitometric tracings of the cell surface glycoproteins from NaB^3H_4-labeled MLC T blasts before and after enrichment for Ly 1^+2^- and Ly 1^-2^+ blasts. Purified C57BL/6 anti-DBA/2 MLC T blasts were dissected into Ly 1^+2^- and Ly 1^-2^+ blasts by treatment with the respective anti-Ly antisera plus complement. The individual groups of Ly phenotype-enriched blasts were surface-labeled with NaB^3H_4, solubilized, and prepared for electrophoresis. Samples were adjusted to a total of 12,500 cpm (5-10 μg protein) and run in a linear 7.5-10% acrylamide gradient containing 0.1% SDS.

culture. Thus, there exists a positive correlation both in the time and extent of T 145 expression with that of cytolytic activity by Con A-induced lymphoblasts.

The Selective Expression of T 145 on Ly $1^-2^+3^+$ Lymphoblasts. Murine T lymphoblasts can be subdivided according to Ly 1,2,3 phenotype into three well-defined groups: Ly $1^+2^+3^+$, Ly $1^+2^-3^-$, and Ly $1^-2^+3^+$ blasts. As mentioned previously, this phenotypic variation is at least in part correlated with functional diversity. To further investigate the expression of T 145 with respect to Ly phenotype, C57BL/6 anti-DBA/2 blasts were generated in MLC so that the resulting blasts would represent activations against the entire *H-2* complex and Mls. At the peak of the proliferative response, blasts were isolated by 1 *g* velocity sedimentation, divided, and incubated with PBS or an excess of anti-Ly 1 or 2 specific antisera for 45 min on ice. At the end of this incubation, the cells were washed once in culture media, resuspended with appropriately diluted, absorbed rabbit complement (21), and incubated for an additional period of 30 min at 37°C. The cells were then diluted to 1 ml with ice-cold media, and cell debris was removed by centrifugation through FCS (22). The various groups of cells were then washed twice with PBS before surface labeling with NaB^3H_4. After labeling and solubilization, the surface glycoprotein patterns from the original T lymphoblasts, as well as the populations enriched for Ly 1^+2^- or Ly 1^-2^+ blasts, were then analyzed by SDS-polyacrylamide gel electrophoresis. As seen in Fig. 4, analysis of the densitometric tracings of the surface glycoproteins from the unfractionated and respective Ly-enriched blasts revealed a complete absence of T 145 on Ly 1^+2^- blasts, which could be seen to be an exclusive surface marker by Ly 1^-2^+ blasts.

Whether or not such a specific enrichment for Ly 1^-2^+ T cells before activation

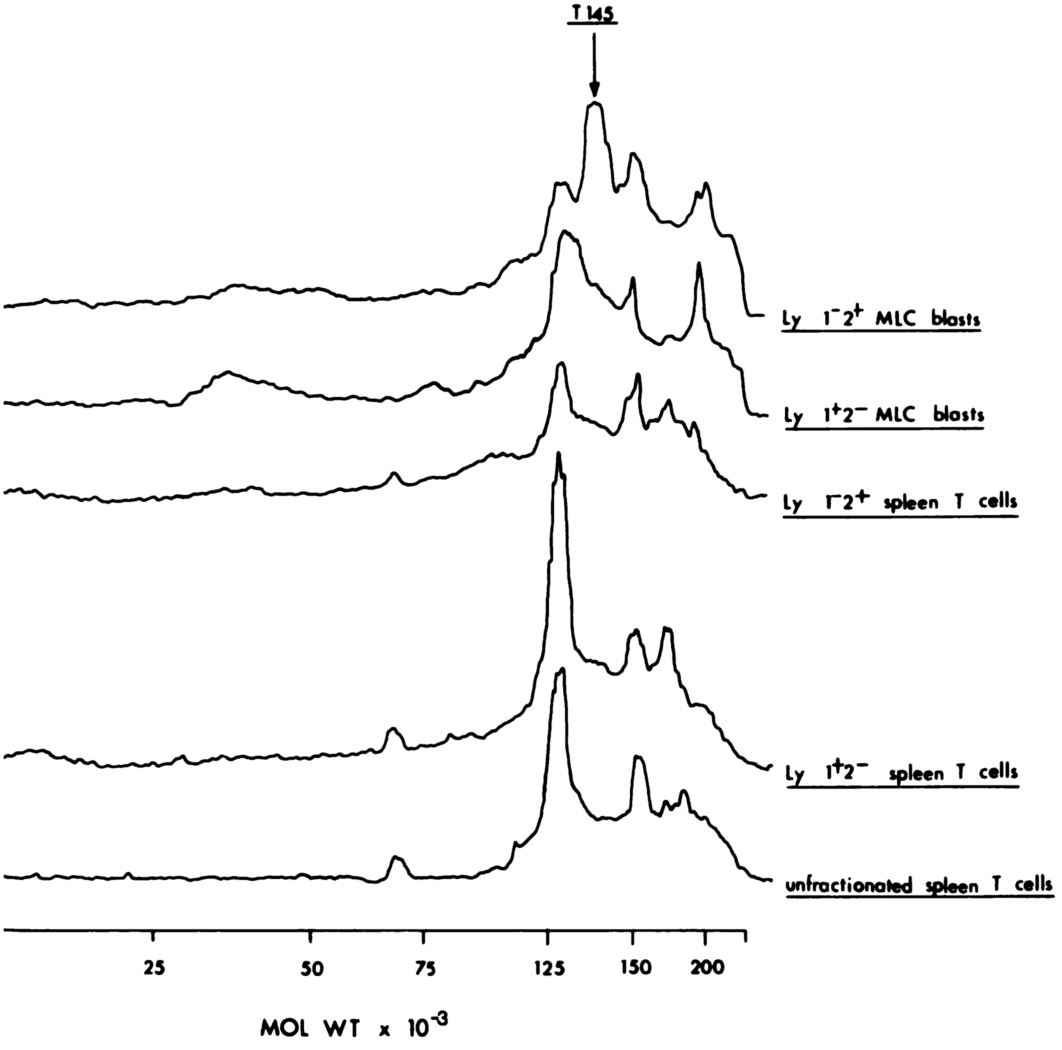

FIG. 5. Densitometric tracings of the cell surface glycoproteins of nonimmune spleen T cells before and after enrichment for Ly 1^+2^- and Ly 1^-2^+ subpopulations of T cells. Ig-anti-Ig column-purified spleen T cells from C57BL/6 mice were treated twice with the respective anti-Ly antisera plus complement before surface labeling with NaB^3H_4. Samples were solubilized and adjusted to a total of 15,000 cpm (5–10 μg protein) and compared with the corresponding Ly-enriched populations of MLC blasts by electrophoresis through a linear 7.5–10% gradient of acrylamide containing 0.1% SDS.

would also reveal T 145 was then examined. C57BL/6 spleen cells were first purified by passage through Ig-anti-Ig-coated columns and divided into three groups as above. To ensure maximum enrichment of the respective Ly subclasses of T cells, each group was treated twice with antiserum and complement. After removal of the dead cells, the cells were labeled with NaB^3H_4, solubilized, and prepared for electrophoresis. As seen in Fig. 5, a 10-fold enriched population of Ly 1^-2^+ cells failed to show any significant expression of T 145. These results further support the contention that T 145 arises through a maturation process

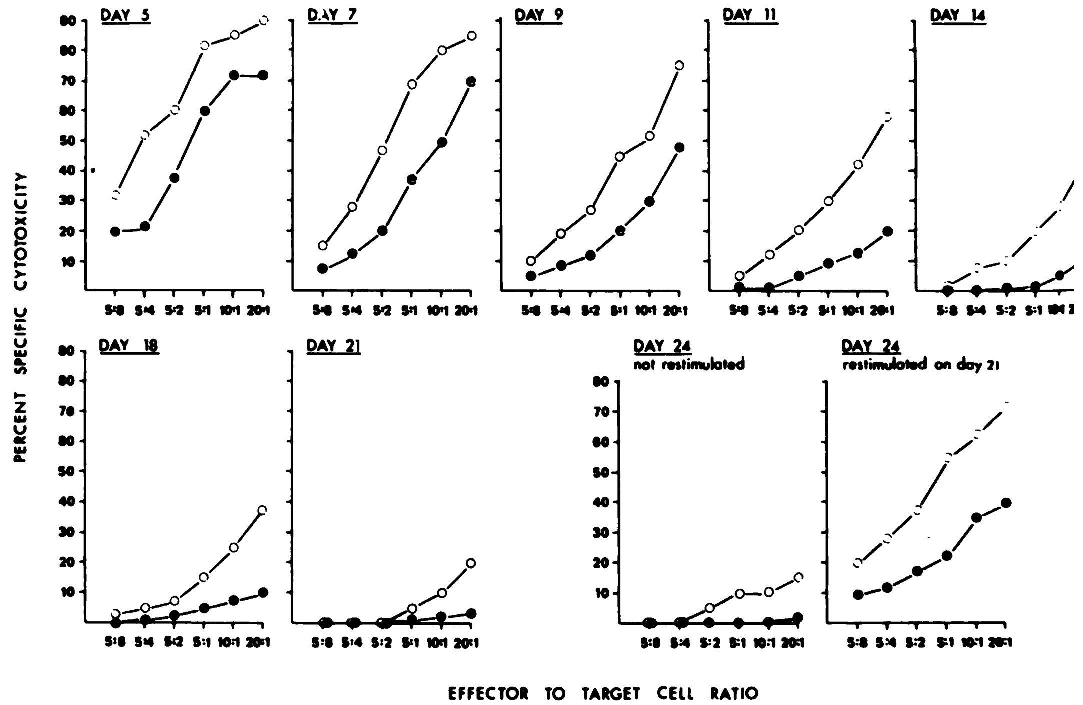

FIG. 6. Cytolytic titration curves of CBA/H anti-DBA/2 MLC T blasts during their morphological reversion to small lymphocytes. MLC blasts were harvested from culture on day 5, purified by 1 *g* velocity sedimentation through a linear 15-30% FCS gradient, and returned to culture at a concentration of 5 × 10^5 cell/ml on syngeneic macrophage feeder layers. At the indicated times, cells were tested for their cytolytic activity against ^{51}Cr-labeled target cells at a variety of effector to target cell ratios in both a 2-h (●) and 4-h (○) assay.

of Ly 1^-2^+ T cells at a time concomitant with the expression of cytolytic activity of these cells.

The T 145 Glycoprotein is a Differentiation Molecule Typical of a Restricted, Primed Subset of T Lymphocytes. The stability of T 145 in the absence of antigenic or mitogenic stimulation could be expected to further distinguish it as either a blast-stage-restricted marker or a more permanent type differentiation antigen, unrelated to the blast state or functional activity of the cell per se. To examine this question, a large number of MLC-activated T lymphoblasts (CBA/H anti-DBA/2) were isolated at the peak of the primary cytotoxic response and allowed to undergo morphological and functional reversion on syngeneic macrophage feeder layers (23). At various times during the reversion process, samples of cells were analyzed for cytolytic ability, percentage of blasts, and the presence of the T 145 glycoprotein on their surface.

The results relating to the functional activity of these cells are shown in Fig. 6. As seen, the peak of cytolytic activity occurs on day 5, followed by a relatively steady but slow decrease during the next 16 days in culture, reaching low yet significant levels on day 21. In contrast to this relatively slow decline in cytolytic activity, morphological reversion to small lymphocytes is essentially complete by day 11-12 (97% small lymphocytes). That quiescent cytotoxic

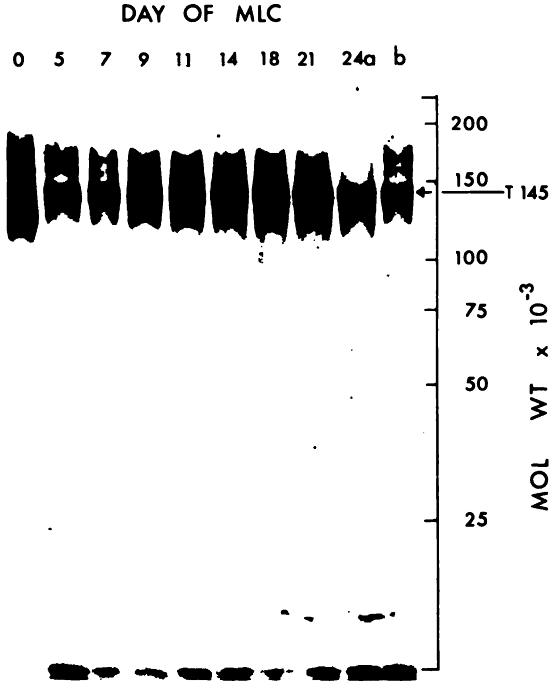

FIG. 7. Cell surface glycoprotein patterns of MLC T blasts during their morphological reversion to small lymphocytes. Aliquots of cells tested for cytolytic activity in Fig. 6 were taken on the indicated days of culture and surface-labeled with NaB^3H_4. 24a represents the glycoprotein banding patterns of cells harvested on the 24th day of culture and 24b represents the glycoprotein pattern of a day 21 culture restimulated with irradiated DBA/2 spleen cells and harvested 3 days later. Samples were adjusted to a total of 15,000 cpm (5-10 μg protein) and separated by electrophoresis through a linear 7.5-10% gradient of acrylamide containing 0.1% SDS.

memory cells were indeed represented in the day 21 population is shown by the positive control in which a portion of these cells could be shown to display a rapid, strong increase in cytotoxic activity upon restimulation with the relevant irradiated stimulator cells (23).

The cell surface glycoprotein patterns of cells taken at various times during this reversion process and labeled with NaB^3H_4 are seen in Fig. 7. From this figure it is clear that once induced, T 145 is expressed with seemingly equal intensity on blasts and small reverted T lymphocytes. We would thus conclude that within the limited time span studied, T 145 behaves as a permanent type differentiation structure whose expression is not restricted to the blast stage of activation. The question of the involvement of T 145 as a possible "killing-relevant" structure could not be answered by these experiments.

Discussion

T lymphocytes are known to display a group of unique surface molecules which have been determined in part by serology (1, 24, 25), and by biochemical approaches (11, 17, 26, 27). Some of these molecules are only found on the T lymphocytes at a certain stage of differentiation (25), whereas others would seem to be maintained throughout differentiation (1, 24). In the present study, we could confirm and extend earlier findings on the characterization of T-cell surface glycoproteins and their distinction from those expressed on B lymphocytes (11, 17). Our main interest, however, has been focused upon the characterization of a unique membrane glycoprotein, absent on resting T lymphocytes and simultaneously expressed on T blasts with the development of effector cell function. We have called this glycoprotein T 145 because of its exclusive expression on T cells and its apparent molecular weight in SDS gels of 145,000 daltons.

Studies on the cellular distribution of T 145 have consistently shown that B lymphocytes, B blasts, and B lymphomas lack this glycoprotein. In the same way, resting T lymphocytes and all 15 T lymphomas analyzed thus far have also been negative. Additional studies with mouse peritoneal exudate macrophages, erythrocytes, and fibroblasts have also shown these cell types to be negative. Thus, the cellular distribution pattern of T 145 indicates that this protein is a unique surface marker of T cells with restricted expression on T lymphoblasts.

Through an analysis of various alloantigenic barriers in the mouse known to induce a primary MLC in vitro, we have been able to assign a ranking order of efficiency for the induction of T 145^+ blasts, based on the genetic incompatibility between the responding and stimulating cell type. Here we could demonstrate that blasts activated against SD determinants of the *H-2D* or *K* regions expressed the highest amount of radioactivity associated with T 145 (Table I). T-cell activations involving genetic differences across the entire *H-2* complex ranked second in efficiency for the generation of T 145^+ blasts, followed by activations against *I* region only. Blasts generated in several syngeneic, Mls-incompatible combinations have been the poorest in T 145 expression of the genetic activations studied.

In addition, we have used Con A as an antigen-independent system of activation to induce T blasts of a wider range of immunological activities (5). Through the use of previously described techniques to measure the total cytotoxic potential of mitogen-activated T cells (10) we have been able to directly correlate both the appearance in time and the extent of T 145 expression with the generation of cytolytic activity by these cells. Additional studies have now shown that PHA-induced blasts generate lower but significant levels of cytotoxicity, and that the time and extent of T 145 expression on these blasts correlate in a highly significant way with cytolytic activity. In both systems of mitogen activation, the expression of T 145 did not parallel the initial state of blast transformation, but was instead concomitant with the appearance of cytotoxic function by these cells.

As the anti-SD reactive T cells are known to constitute better killer cells than the anti-Ia and anti-Mls cells (1, 18, 19), the relative intensity of T 145 expression seemed to correlate well with the levels of cytolytic activity induced by the various genetic systems (Table I), and to account for the higher expression on Con A vs. PHA blasts (28). Aside from being functionally distinct, anti-SD reactive T cells are normally characterized by the serological phenotype Ly 1^-2^+, whereas the anti-Ia cells are mostly Ly 1^+2^- (1). Thus, in striking accordance with the expectation that T 145 may represent a marker for killer T cells, were the findings that Ly 1^+2^- blasts were T 145^-, and that Ly 1^-2^+ blasts were strongly T 145^+. Suppressor T cells may also express an Ly phenotype similar to that of killer T cells, but treatment of MLC blasts with antisera raised against I-J (29) in the presence of complement does not eliminate T 145-positive cells in any detectable degree (unpublished observations). These findings do not exclude the possible expression of T 145 on suppressor T cells, but they would argue strongly against T 145 as constituting a marker for suppressor cells only.

Both the tissue distribution (1) and current molecular weight estimations of 35,000 daltons (30) make it highly unlikely that T 145 is an Ly 2 or 3 antigen. Furthermore, work in progress involving sequential immune precipitations of lactoperoxidase and NaB^3H_4 surface-labeled T blasts with a variety of alloantisera against known antigenic systems distinguish T 145 as a unique surface molecule.

The stability of T 145 on killer T lymphoblasts undergoing morphological reversion to small, poorly cytotoxic T cells clearly indicates that the expression of this glycoprotein is not restricted to the blast state. On the contrary, the present evidence would indicate that once induced, T 145 expression persists and is no longer dependent upon the presence of stimulating antigen or the blast state.

It is known from the work of others as well as that of our own that killer T cells in the mouse (31, 32), rat (K. Welsh, personal communication), and human[3] express new "killer cell-unique" surface antigens. Although the exact relationship between these findings and T 145 remains to be established, the evidence presented here strongly supports the contention that T 145 is such a unique killer T cell marker. We would at present take the view that T 145 represents a surface glycoprotein appearing on T lymphocytes undergoing immune activation by either a relevant polyclonal T-cell activator like Con A, or by alloantigens in such a way as to yield cytolytic T cells in the resulting cell population.

From internal labeling experiments on highly purified T lymphoblasts we know that the T 145 glycoprotein indeed represents an actual product of the T cell, and that it can serve as an immunogen for antibody synthesis across species barriers.[4] The function of T 145^+ cells may be in toto of cytolytic nature, and the actual presence of T 145 on the surface of such T cells may be a necessary requirement for directed lysis to occur, but could require additional functional activities of the cell to allow the lytic function to become expressed. If this is correct, anti-T 145 antibodies should be unusually efficient inhibitors of cytotoxic T lymphocytes, and preliminary experiments would suggest this to be the case.

In conclusion, we have presented evidence on the appearance of a new surface glycoprotein on immunocompetent T cells appearing at a given stage of differentiation. This protein could be shown to be selectively expressed on the surface of T-cell blasts endowed with cytolytic ability, reacting against SD determinants, and with the phenotype Ly 1^-2^+. Further analysis of this glycoprotein should now allow deeper insight as to how these specialized T lymphocytes exert their biologically relevant functions.

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[4] A. Kimura and H. Wigzell. Manuscript in preparation.

Summary

T lymphocytes at various stages of maturation and differentiation have been isolated by cellular fractionation procedures and characterized by cell surface markers and functional assays. The cell surface glycoproteins of the various T-cell preparations have been selectively radiolabeled by the galactose oxidase-tritiated sodium borohydride technique and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography.

Details are presented on the appearance of a new cell surface glycoprotein (T 145), present on immunocompetent T lymphocytes after activation by either major histocompatibility complex alloantigens or by concanavalin A. The intensity of T 145 expression on T lymphoblasts is shown to be directly correlated in time and extent to the levels of cytotoxicity generated in a variety of T-cell activations.

Specific enrichment procedures of purified populations of mixed leukocyte culture blasts have shown Ly 1^+2^- blasts to be T 145^- and Ly 1^-2^+ blasts to be strongly T 145^+. Similar enrichment procedures on normal peripheral T cells have failed to reveal any significant expression of T 145 on a highly enriched population of Ly 1^-2^+ T cells. Further studies on the stability of T 145 expression after induction have shown it to be a more permanent-type differentiation structure whose expression is clearly not linked to the blast stage of activation.

T 145 would thus appear to represent a membrane glycoprotein whose exclusive expression on T lymphoblasts is further restricted to a defined group of cells endowed with cytolytic activity and bearing the Ly phenotype Ly 1^-2^+.

We wish to thank Dr. J. Andersson for the generous supply of purified IgG_1 myeloma proteins and mitogens used in these studies, and Dr. E. A. Boyse for supplying us with anti-Ly sera. The skillful secretarial assistance of Birgitta Ehrsson is gratefully acknowledged.

Received for publication 18 October 1977.

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PRIMARY IN VITRO CELL-MEDIATED LYMPHOLYSIS REACTION OF NZB MICE AGAINST UNMODIFIED TARGETS SYNGENEIC AT THE MAJOR HISTOCOMPATIBILITY COMPLEX*

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NZB mice develop a progressive autoimmune disease resembling human systemic lupus erythematosus (1-3) in many respects. The natural history of this disease is characterized by the development of hemolytic anemia, splenomegaly, glomerulonephritis, and numerous autoantibodies (4-5). Humoral immune responses to a number of antigens have been found to be abnormal in NZB mice (4-8) and induction of tolerance to soluble protein antigens to be more difficult in NZB than in other strains (9). A variety of mechanisms have been proposed for these abnormalities (10-14). In older mice, T-cell functions like mitogen responsiveness (15), graft-versus-host reactivity (16), and cell-mediated cytotoxicity (17-20) have generally been decreased, although in a recent report (21) the level of some of these functions in the NZB strain has been observed to be within the range of normal.

In previous investigations (17-21) the phenomenon of cell-mediated lympholysis (CML)[1] in NZB mice has been examined by using targets differing at the major histocompatibility complex (MHC) from NZB or against virus-infected targets. In the experiments reported here, the primary in vitro CML activity of NZB mice was investigated against syngeneic targets and against targets carrying the NZB MHC type ($H\text{-}2^d$) on a background differing from NZB. In this situation primary in vitro CML activity is not generated by normal strains (22-26). In the present experiments a significant unidirectional CML was unexpectedly demonstrated against *H-2* identical allogeneic targets by NZB effector cells. These results represent the first demonstration of a CML reaction in a primary in vitro system directed against unmodified targets which do not differ from the cytotoxic effectors in the MHC. They provide evidence for a qualitative difference in T-cell cytotoxic function between NZB mice and control

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* Supported by U.S. Public Health Service Research grant no. AM-18505.

‡ Recipient of training grant no. BO544/1 of the Deutsche Forschungsgemeinschaft.

§ Recipient of Research Career Award, National Institute of Health.

[1] *Abbreviations used in this paper:* CML, cell-mediated lympholysis; Con A, concanavalin A; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; LPS, lipopolysaccharide; MHC, major histocompatibility complex; MIH, minor histocompatibility complex; PHA, phytohemagglutinin; UTHSCD, The University of Texas Health Science Center at Dallas.

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strains. The capacity to respond in this system against H-2 identical targets may be related to the autoimmune disease of NZB mice.

Materials and Methods

Mice. Unless otherwise stated, 3-4 mo old male mice were used throughout the study. NZB mice were obtained from the breeding colony of the Rheumatic Disease Unit of the University of Texas Health Science Center at Dallas (UTHSCD), originally obtained from Dr. Marianne Bielschowsky, the University of Dunedin, New Zealand. BALB/c, C57BL/6, C57BL/10, B10.D2, and DBA/2 mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. B6.C-H-2^d mice (HW19) were a generous gift of Doctors James Forman and Donald Bailey; all other strains used were bred and maintained in the colonies of J. K. at the UTHSCD.

Skin Grafting. Skin grafts were performed following the method described elsewhere (27).

Serological Testing. Serological tests for the presence of transplantation antigens were done according to Hauptfeld et al. (28).

Culture Media. Hanks' balanced salt solution (HBSS) (Microbiological Associates, Walkersville, Md.) was used for washings. RPMI-1640 (Grand Island Biological Company, Grand Island, N.Y.) supplemented with 2 mM 1-glutamine (Gibco), 100 U/ml penicillin, 0.25 μg/ml Fungizone, 100 mcg/ml streptomycin (Gibco), 5×10^{-5} M 2-mercaptoethanol (Eastman Kodak Co., Rochester, N.Y.), fetal calf serum (Gibco) 10% and 25 mM Hepes buffer (Gibco) was used for all cultures.

Cell Suspensions. Mice were sacrificed by cervical dislocation and their spleens aseptically removed. The spleens were gently teased apart in cold HBSS and the resulting suspension aspirated through a 24-gauge needle. Clumps were allowed to settle for 5 min and removed. The suspension was washed three times in cold HBSS, and after the third wash the cells were resuspended in RPMI-1640 and counted in a Coulter Counter, (Coulter Electronics, Inc., Hialeah, Fla.). All suspensions were adjusted to 4×10^6 cells/ml. Viability, as checked by trypan blue exclusion, was more than 95%.

Stimulating Cells. Stimulator cells were irradiated with 3,000 rads (cesium source, 100 rads/min).

Preparation of Effector Cells. $^1/_2$ ml of responding cells (2×10^6 cells) and 0.5 ml of stimulator cells (2×10^6 cells) were placed in 35 × 10 mm polystyrene Petri dishes (Corning, no. 25,000, Corning Glass Works, Science Products Div., Corning, N.Y.). Each effector-stimulator combination was set up in triplicate. The dishes were incubated in an atmosphere of 83% N_2, 10% CO_2, and 7% O_2 at 37°C on a rocking platform for 5 days (29). On the 5th day, target cells were added.

Preparation of Target Cells. 4 ml of the spleen cell suspensions containing 16×10^6 cells were incubated in Falcon 3013 flasks (BioQuest, BBL & Falcon Products, Becton, Dickinson & Co., Cockeysville, Md.) at 37°C in an atmosphere of 5% CO_2 in air for 5 days. 48 h after the beginning of the culture, 5 μg/ml concanavalin A was added. On the 5th day, the resulting blast cell suspension was washed in HBSS and 200-400 μCi ^{51}Cr-sodium chromate in 0.2 ml saline and 50 μl FCS was added to the pellet. The cells were resuspended and incubated for 1 h at 37°C with occasional shaking. The cell suspension was then washed with HBSS, resuspended in 1 ml of medium, and layered on a Ficoll-Hypaque solution with a sp gr of 1.077 (Isolymph, Garrard-Schlesinger, Carle Place, N.Y.). The gradient was spun at 1,200 rpm for 10 min at 4°C. The cells at the interface were harvested with a Pasteur pipette and washed two times in HBSS, resuspended in medium, and counted. Viability of the cells was between 85 and 98% with most cells having a large blastoid-like appearance. The suspension was then adjusted to 1×10^5 cells/ml.

CML Assay. 10^4 target cells were added to each effector cell culture, and cultures were incubated for 4 h at 37°C in the gas mixture (83% N_2, 10% CO_2, 7% O_2). Thereafter, the content of the Petri dishes was transferred to 12 × 75-mm glass tubes and 2 ml of cold saline was added to stop the cytotoxic reaction. The tubes were shaken and spun down at 2,000 rpm for 5 min at 4°C. 1 ml of the supernate was transferred to another tube and the radioactivity in both corresponding tubes was counted in a gamma counter (Packard 5230, Packard Instrument Co., Inc., Downers Grove, Ill.). The counts were corrected for the counter background counts and ^{51}Cr release was calculated as percent of the total label incorporated according to the formula:

$$\%\ \text{release} = \frac{(\text{cpm}\ ^1/_3\ \text{supernate}) \times 3}{(\text{cpm pellet} + \text{cpm}\ ^2/_3\ \text{supernate}) + (\text{cpm}\ ^1/_3\ \text{supernate})} \times 100.$$

Specific lysis was defined as the total lysis in a culture minus the background lysis of the target cells lysed. Background lysis was defined in every experiment as the release from target cells which were added to cultures of autologous effector and autologous stimulator cells. Additionally, control cultures of effector cells with allogenic stimulator cells tested against targets autologous with the effectors were set up.

Dose-Response Experiments. Effector cell cultures were pooled on the 5th day and the cell suspension washed two times in HBSS. The number of viable cells was determined by trypan blue exclusion. The desired number of potential effectors and 10^4 target cells in a total vol of 1 ml of fresh medium were placed into Falcon 2054 tubes, centrifuged at 300 rpm for 2 min, and then incubated for 4 h at 37°C in an atmosphere of 5% CO_2 in air. After 4 h, 2 ml of cold saline was added and ^{51}Cr release determined.

Anti-Thy 1 Treatment. Effector cell populations were pooled and washed two times in HBSS. The suspension was divided into two parts. One part was incubated with normal rabbit serum for 30 min in an ice bath and the other part with a rabbit anti-mouse brain serum. This serum was a gift of Dr. J. Cambier of the Department of Microbiology UTHSCD. Its production and characteristics have been described elsewhere (30). After 30 min, both the suspensions were washed in HBSS and incubated 30 min at 37°C in guinea pig serum diluted 1:4 with medium. Afterward, the suspensions were washed again two times with HBSS and viable cells were counted. The CML assay was performed in parallel with both suspensions in tubes as described above.

Mitogen Stimulation. 50,000 viable cells were incubated with PHA (Difco Laboratories, Detroit, Mich. 1:500), Con A (Pharmacia, Uppsala, Sweden, 5 μg/ml), or LPS (Difco, *E. coli* 0111:134 10 μg/ml for 2 days in round bottom microtiter plates. 1 μCi of [^{3}H]thymidine was then added to the cultures and after additional 6 h of incubation, the cultures were harvested with an automatic harvester (MASH II, Microbiological Associates) and radioactivity counted in a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

Statistical Calculations. Student's *t* tests were performed by using a program of the Medical Computing Resources Center, UTHSC at Dallas, on a DEC 10 computer.

Results

CML of NZB (H-2^d against BALB/c (H-2^d). Table I shows the results of an experiment, in which CML reactions of NZB mice of two different ages, 3 and 10 mo, against *H-2* identical BALB/c and *H-2* disparate C57BL/6 mice were examined. The NZB mice did not generate CML activity against each other, nor did BALB/c effectors react against NZB targets. In striking contrast, NZB mice, both young and old, generated a significant CML reaction against BALB/c targets. The fact that BALB/c exerted a strong CML reaction against C57BL/6 target cells indicated that the cytotoxic effector function of BALB/c was normal.

To verify the unexpected finding of primary in vitro CML against *H-2* identical targets by NZB effectors, five similar experiments were carried out. The results are summarized in Table II. In none of 12 CML tests of BALB/c effector cells against NZB targets, 6 of these against target cells of young animals and 6 against target cells of old animals, did BALB/c generate specific lysis of more than 10%, the mean specific release of all 12 reactions being 2.0 ± 2.1%. In contrast, 10 of 12 NZB versus BALB/c reactions yielded more than 10% specific lysis. The mean specific release was 26.5 ± 5.1%. The difference between the specific lysis obtained by NZB against BALB/c and that of BALB/c against NZB, is highly significant ($P < 0.001$, Student's *t* test). No significant CML reactivity was observed between old and young NZB.

Dose-Response Experiments. To establish that the NZB anti-*H-2^d* cytotoxic effect was caused by cells and not by a transferred soluble factor, two dose-response experiments were done. (Fig. 1) In the first experiment we investigated

TABLE I

Primary in Vitro CML of NZB Mice against BALB/c

Effector	Stimulator	Target	Release[*]	Specific release
			%	%
BALB/c	BALB/c	BALB/c	24.4 ± 2.2	–
C57BL/6			73.6 ± 1.2	49.2
NZB Old[‡]			53.0 ± 2.3	28.6
NZB Young[§]			55.8 ± 4.2	31.4
BALB/c	C57BL/6	C57BL/6	65.2 ± 1.1	44.8
C57BL/6			20.4 ± 0.6	–
NZB Old			56.0 ± 5.6	35.6
NZB Young			71.9 ± 1.2	51.5
BALB/6	NZB Old	NZB Old	34.5 ± 2.0	2.3
C57BL/6			66.8 ± 4.0	34.6
NZB Old			32.2 ± 1.3	–
NZB Young			42.5 ± 3.8	10.3
BALB/c	NZB Young	NZB Young	35.4 ± 3.8	7.1
C57BL/6			76.9 ± 1.2	48.6
NZB Old			27.2 ± 0.5	−1.1
NZB Young			28.3 ± 1.7	–

[*] Mean and standard error of the mean of triplicate determinations.

[‡] 10 mo old.

[§] 3 mo old.

TABLE II

Primary in Vitro CML of NZB Mice of Different Ages against BALB/c

Effector	Stimulator	Target	Specific release[*]	
			%	
C57BL/6	BALB/c	BALB/c	52.4 ± 4.9	
BALB/c	C57BL/6	C57BL/6	46.5 ± 1.6	
C57BL/6	NZB Old[‡]	NZB Old	40.0 ± 3.9	
C57BL/6	NZB Young[§]	NZB Young	42.2 ± 5.5	
NZB Old	BALB/c	BALB/c	22.8 ± 4.9	$P < 0.005$[‖]
BALB/c	NZB Old	NZB Old	4.5 ± 1.1	
NZB Young	BALB/c	BALB/c	30.3 ± 6.2	$P < 0.001$[‖]
BALB/c	NZB Young	NZB Young	0.3 ± 3.9	
NZB Old	NZB Young	NZB Young	−1.0 ± 2.2	
NZB Young	NZB Old	NZB Old	7.2 ± 2.4	

[*] Mean and standard error of the mean of the specific release in six experiments.

[‡] 9-11 mo of age.

[§] 2-4 mo of age.

[‖] Student's *t* test.

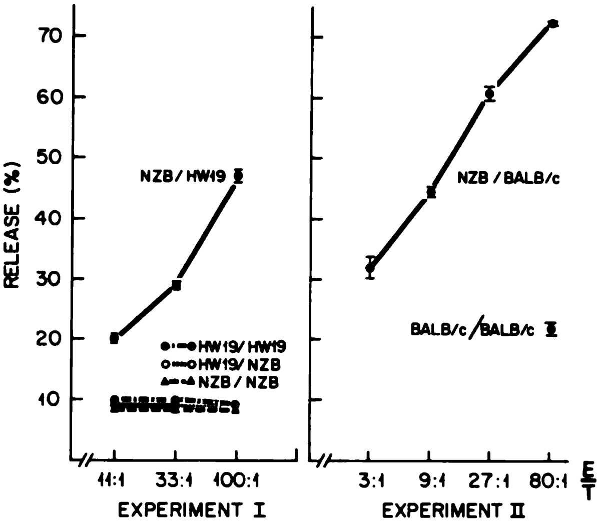

FIG. 1. Dependency of the NZB anti-H-2^d reaction on the effector/target cell ratio.

the reaction of NZB against HW19 (a congenic line, carrying H-2^d, derived from BALB/c on a C57BL/6 background); in the second experiment the NZB anti-BALB/c reaction was tested. The amount of label released from a constant number of targets (10^4) in both experiments was clearly dependent on the number of viable effector cells added, indicating that the cytotoxic effect was exerted by cells.

CML of NZB against Other H-2^d Strains. In subsequent experiments, the CML reactions of NZB against other H-2^d strains were tested. Table III gives a representative experiment of this series. As expected, no significant specific release was observed in primary in vitro cultures set up between the normal H-2^d carrying strains DBA/2, B10.D2, and BALB/c. Also, none of these strains reacted against NZB, although they did react with the H-2 different target C57BL/10. In contrast, as observed above with the BALB/c target, NZB cells reacted against all tested H-2^d strains, indicating that the unidirectional CML activity of NZB against H-2^d targets was not restricted to the BALB/c H-2^d. These results were confirmed in two further experiments by using the strain HW19 in addition to the other strains.

Cross-Reactivity of NZB Effector Cells on Different H-2^d Targets. The specificity of the NZB effector cells obtained after BALB/c H-2^d primary in vitro sensitization was investigated in the experiment shown in Table IV. NZB effectors, sensitized with BALB/c cells, were tested against BALB/c, DBA/2, B10.D2, and C57BL/10 targets. Significant specific lysis of all three H-2^d carrying targets was observed, indicating cross-reactivity in this respect. A small reaction against C57BL/10 was observed, raising the question of cross-reactivity with a non-H-2 identical target. This is examined below. Sensitization with C57BL/10 stimulators as a control resulted in lysis only of the appropriate target. In additional experiments, NZB effector cells sensitized with DBA/2 or B10.D2 showed the same H-2^d cross-reactions.

TABLE III

Primary in Vitro CML of NZB against Three Different H-2^d Strains

Effector	Stimulator	Target	Release*	Specific release
			%	%
C57BL/10	C57BL/10	C57BL/10	23.7 ± 2.3	–
B10.D2			66.7 ± 1.6	43.0
BALB/c			63.1 ± 1.2	39.4
DBA/2			63.5 ± 3.5	39.8
NZB			68.2 ± 3.0	44.4
B10.D2	NZB	NZB	24.4 ± 2.2	2.8
BALB/c			17.6 ± 1.6	–4.0
DBA/2			22.7 ± 1.9	1.1
NZB			21.6 ± 0.2	–
B10.D2	B10.D2	B10.D2	17.0 ± 1.9	–
BALB/c			18.6 ± 2.3	1.6
DBA/2			22.0 ± 1.0	5.0
NZB			52.1 ± 1.6	35.0
B10.D2	BALB/c	BALB/c	22.5 ± 2.6	3.0
BALB/c			19.5 ± 0.3	–
DBA/2			25.2 ± 0.5	5.7
NZB			75.2 ± 1.6	55.7
B10.D2	DBA/2	DBA/2	25.4 ± 2.3	2.7
BALB/c			22.5 ± 1.9	–0.2
DBA/2			22.7 ± 1.2	–
NZB			68.9 ± 8.2	46.2

* Mean and standard error of the mean of triplicate determinations.

TABLE IV

Cross-Reactivity of NZB CML Effectors Sensitized by BALB/c on Different H-2^d Targets

Effector	Stimulator	Target	Specific release
			%
NZB	BALB/c	BALB/c	29.1
NZB	C57BL/10	BALB/c	3.4
NZB	BALB/c	DBA/2	21.8
NZB	C57BL/10	DBA/2	6.3
NZB	BALB/c	B10.D2	19.5
NZB	C57BL/10	B10.D2	–1.0
NZB	BALB/c	C57BL/10	10.2
NZB	C57BL/10	C57BL/10	45.7
NZB	BALB/c	NZB	–1.9
NZB	C57BL/10	NZB	–2.5

TABLE V

Cross-Reactivity of NZB CML Effectors on the Congenic Lines B10 and B10D2

Effector	Stimulator	Target	Release*	Specific release
			%	%
C57BL/10	C57BL/10	C57BL/10	25.4 ± 0.8	–
C57BL/10	B10.D2		22.1 ± 0.5	−3.3
B10.D2	C57BL/10		76.2 ± 2.9	50.8
NZB	C57BL/10		79.5 ± 2.4	54.1
NZB	B10.D2		41.8 ± 11.2	16.4
C57BL/10	B10.D2	B10.D2	57.8 ± 3.4	37.0
C57BL/10	NZB		81.9 ± 1.9	60.1
B10.D2	C57BL/10		28.5 ± 2.2	7.7
B10.D2	B10.D2		20.8 ± 1.0	–
NZB	C57BL/10		77.4 ± 5.1	56.6
NZB	B10.D2		65.8 ± 6.3	45.0
B10.D2	C57BL/10	NZB	15.0 ± 0.8	−2.9
B10.D2	NZB		19.9 ± 1.8	2.0
NZB	C57BL/10		19.1 ± 1.3	1.2
NZB	B10.D2		21.7 ± 1.0	3.8
NZB	NZB		17.9 ± 1.5	–

* Mean and standard error of the mean of triplicate determinations.

Confirmation of the Presence of all $H\text{-}2^d$ Transplantation Antigens in NZB. One possible explanation of the unidirectional cross-reactive CML response of NZB against $H\text{-}2^d$ targets would be that other $H\text{-}2^d$ strains possess $H\text{-}2^d$ antigens which are lacking in NZB. NZB would then recognize such antigens as foreign and react against cells expressing the complete set of $H\text{-}2^d$ antigens. To establish the presence of all transplantation antigens in the NZB $H\text{-}2^d$ complex, an F_1 hybrid test was used. 10 (NZB × C57BL/10) F_1 hybrids were grafted with B10.D2 skin. No graft rejection occurred during the observation period of 100 days. Furthermore, serological tests for the presence of the $H\text{-}2^d$ antigens *H-2* and *Ia* were carried out by the direct cytotoxic test (28) and by the absorption method (28). All tested $H\text{-}2^d$ antigens were found to be expressed on NZB cells. So none of the antigens of the MHC, as defined either by the F_1 hybrid test or serologically is missing in NZB.

Cross-Reactivity of NZB Anti-$H\text{-}2^d$ Effectors on $H\text{-}2^b$ Targets. Another possible explanation of the NZB anti $H\text{-}2^d$ CML is that the reaction is directed against minor histocompatibility (H) antigens recognized in the context of the $H\text{-}2^d$ haplotype. If so, the reaction would be $H\text{-}2^d$ restricted. Whether there is an *H-2* restriction of the cytotoxic effectors in NZB was investigated in experiments utilizing two pairs of congenic lines: C57BL/10 ($H\text{-}2^b$) B10.D2 ($H\text{-}2^d$), and C57BL/6 ($H\text{-}2^b$) and HW19 ($H\text{-}2^d$). The members of both pairs differ only in the *H-2* region. If NZB cells sensitized with one congenic partner were to lyse targets of both congenic partners, the CML would not be *H-2* restricted. If, on the other hand, only the *H-2* identical target were to be lysed, the *H-2* complex would be implicated in the reaction. Table V gives the result of a representative experiment and Table VI the summary of three experiments.

TABLE VI

Cross-Reactivity of NZB CML Effectors on the Congenic Lines C57BL/10 and B10.D2: Summary of Three Experiments

Effector	Stimulator	Target	Specific* release
			%
NZB	C57BL/10	C57BL/10	50.3 ± 6.4
NZB	C57BL/10	B10.D2	36.0 ± 12.5
NZB	B10.D2	B10.D2	34.3 ± 8.7
NZB	B10.D2	C57BL/10	15.3 ± 6.2
B10.D2	NZB	NZB	0.8 ± 1.3

* Mean and standard error of the mean of the specific lysis in three experiments.

TABLE VII

Effect of Anti-Thy 1 Serum Treatment on NZB Cytotoxic Reactions

Effector	Stimulator	Target	Incubated	Specific lysis
				%
NZB	C57BL/10	C57BL/10	NRS	55.6
NZB	C57BL/10	C57BL/10	Anti-Thy-1	−2.3
NZB	B10.D2	C57BL/10	NRS	23.1
NZB	B10.D2	C57BL/10	Anti-Thy-1	1.5
NZB	C57BL/10	B10.D2	NRS	60.4
NZB	C57BL/10	B10.D2	Anti-Thy-1	5.9
NZB	B10.D2	B10.D2	NRS	30.6
NZB	B10.D2	B10.D2	Anti-Thy-1	5.3

After sensitization of NZB effectors with C57BL/10, lysis is observed not only of the specific target, C57BL/10, but also of B10.D2 targets. After sensitization with B10.D2 stimulators, lysis not only of B10.D2 but also of C57BL/10 targets occurred. Similar findings were obtained in the reactions involving the congenic combination C57BL/6 and HW19. These results suggest that NZB effector cells in contrast to other strains may not be *H-2* restricted in their cytotoxic aggression. It should be pointed out, however, that the degree of lysis in the cross-reactive reactions was smaller than in the specific reactions.

T-Cell Character of the Cytotoxic Effector Cell. To determine whether cytotoxic T cells were responsible for the cytotoxic reactions demonstrated above, NZB effector sensitized either with C57BL/10 or B10.D2 were incubated with an anti-Thy 1 serum. After incubation in guinea pig serum as complement source, the effector populations were tested in an effector-to-target ratio of 35:1 on C57BL/10 and on B10.D2 targets. Additionally, the sensitized and the anti-Thy-1-treated sensitized populations were stimulated with the mitogens PHA, Con A, and LPS. Table VII gives the results of the CML tests. All CML reactions whether *H-2* allogeneic, NZB anti-H-2^d, or cross-reactive, were totally abolished

TABLE VIII

Selective T-Cell Elimination by Anti-Thy 1 Antiserum Treatment as Judged by Mitogen Stimulation

Mitogen	Treatment	cpm ± SE*
None	NRS	5,068 ± 548
None	Anti-Thy-1	1,810 ± 328
PHA	NRS	17,926 ± 1,782
PHA	Anti-Thy-1	2,169 ± 139
Con A	NRS	9,003 ± 278
Con A	Anti-Thy-1	2,139 ± 169
LPS	NRS	18,336 ± 2,121
LPS	Anti-Thy-1	22,710 ± 1,356

* Mean and standard error of the mean of triplicate determinations.

TABLE IX

*Cross-Reactivity of Cell-Mediated Lympholysis in NZB Mice**

Target		Effectors	
		BALB/c Sensitized with B10.D2	NZB Sensitized with B10.D2
C57BL/10	(H-2^{b})	−0.7	7.8
A.BY	(H-2^{b})	−0.9	9.1
B10.D2	(H-2^{d})	−2.0	42.0
DBA/2	(H-2^{d})	−5.9	27.9
B10.M	(H-2^{f})	−6.9	14.1
A.CA	(H-2^{f})	−0.4	3.4
B10.BR	(H-2^{k})	−6.3	9.2
CBA	(H-2^{k})	−1.3	12.1
B10.Q	(H-2^{q})	−0.5	10.4
DBA/1	(H-2^{q})	2.2	34.0
B10.S	(H-2^{s})	−2.6	19.1
A.SW	(H-2^{s})	−2.2	33.7

* Lysis of targets (*a*) identical to the stimulating cells, (*b*) only *H-2* identical, (*c*) only background identical, and (*d*) nonidentical. Numbers represent specific lysis in percent.

by the anti-Thy 1 treatment. The surviving cells in the antiserum treated population showed normal responses to LPS (Table VIII). These results clearly indicate that the effector cell in the CML reactions described is a T cell.

Cross-reactivity of NZB Effectors on Other B10 Lines and on Targets Allogenic in the H-2 Region and in the Background. The experiment shown in Table IX was done to determine whether NZB effector populations sensitized with *H-2*^{d} cells on the B10 background would cross-react with other congenic

B10 lines expressing other *H-2* haplotypes than $H\text{-}2^d$ and $H\text{-}2^b$, like $H\text{-}2^f$ (B10.M), $H\text{-}2^k$ (B10.BR), $H\text{-}2^q$ (B10.Q), and $H\text{-}2^s$ (B10.S). Additionally, the question of whether NZB effector populations would recognize target cells which share neither the *H-2* haplotype nor the background with the stimulating cell population was investigated. In this experiment, therefore, the CML reactivity of the effector cell population against targets expressing the various *H-2* types on either B10 or non-B10 backgrounds was compared. Throughout the experiment the CML of BALB/c effector cells was assayed in parallel to the NZB effectors as a control. As expected, BALB/c, after sensitization with B10.D2, did not generate CML against any of the target cells used. In contrast, NZB sensitized with B10.D2 again reacted with the B10.D2 target, the sensitizing strain, and the DBA/2 target, carrying the $H\text{-}2^d$ haplotype of the sensitizing cell on a different background. However, there was also reaction with targets such as DBA/1 and A.SW carrying neither the *H-2* haplotype, nor the background of the sensitizing strain. These results indicate that NZB cytoxic effector cells sensitized with B10.D2 have the capacity to cross-react with background antigens of a number of mouse strains carrying different *H-2* haplotypes. They demonstrate that NZB effector cells, after $H\text{-}2^d$ sensitization, display a relatively broad cross-reactivity which is not found in the normal control strain BALB/c. These findings were confirmed in a similar experiment.

Discussion

The experiments reported here demonstrate that NZB mice after primary in vitro sensitization with *H-2* identical cells exert CML activity against target cells syngeneic at the MHC complex. These findings provide evidence for a qualitative T-cell abnormality in NZB mice compared to normal strains which do not generate cytotoxic cells under these conditions. The abnormal reactivity of NZB could be demonstrated as early as 2 mo of age, and was still present at 12 mo of age. These findings indicate that the observed defect is either inherited or acquired early in life and continues after the emergence of the autoimmune disease.

NZB effector cells, sensitized with one $H\text{-}2^d$ strain, exerted CML not only on targets identical to the strain used for sensitization, but also against all other $H\text{-}2^d$ strains tested. However, they did not lyse NZB target cells. In some experiments NZB effectors sensitized with $H\text{-}2^d$ cells cross-reacted with non-$H\text{-}2^d$ targets independently of whether these targets shared the genetic background with the stimulating cell or not. Additionally, NZB anti-$H\text{-}2^b$ effector cells were found to cross-react with $H\text{-}2^d$ carrying target cells bearing the same genetic background. Thus, for example, NZB anti-C57BL/10 effector cells were able to lyse C57BL/10 and B10.D2 target cells.

Several explanations can be considered for the unexpected observation of CML activity of NZB against MHC identical strains and for the cross-reactions described. The first possibility is that the reactions were a nonspecific effect of the in vitro system used. This explanation is ruled out by control experiments involving responding cells derived from strains other than NZB. In all cases, these cells responded with the specificity of the CML reactions as commonly recorded (22).

The second possiblity is that the NZB anti-$H\text{-}2^d$ reactivity noted is an autoimmune response to $H\text{-}2^d$ antigens. Such an explanation is unlikely since the same effector cells which lysed BALB/c targets did not lyse NZB targets. Were the NZB cytotoxic cells reacting with $H\text{-}2^d$ antigens, the NZB targets would have been killed to the same extent as the BALB/c and other $H\text{-}2^d$ targets.

The third possibility is that NZB mice differ from BALB/c and other $H\text{-}2^d$ strains (B10.D2, HW19, DBA/2) in the *H-2* complex and that, for example, the NZB anti-BALB/c reaction is an *H-2* allogeneic reaction. Since the reaction is unidirectional, i.e. BALB/c responders do not kill NZB targets, one would have to presume that BALB/c and other $H\text{-}2^d$ strains carry *H-2* antigens that are absent in NZB. Such an assumption is contradicted by the finding that B10.D2 skin grafts are permanently accepted by (B10 × NZB) F_1 hybrids (31). This observation was reconfirmed by grafting such F_1 hybrids derived by mating mice maintained in our animal colonies. The identity of the *H-2* haplotypes of NZB and B10.D2 is further supported by serological analysis which failed to reveal any difference between the two strains, and by the fact that grafts exchanged between B10.D2 and B10.NZB, a congenic line carrying the *H-2* haplotype of NZB on B10 background, survived indefinitely (P. Ivanyi, personal communication).

The fourth and, in our view, the most likely explanation of the NZB anti-BALB/c reactivity is that the reaction is directed against minor H antigens and as such is *H-2* restricted. As originally demonstrated by Bevan (23) and Gordon and co-workers (24), minor H antigens can be recognized by T cells, but only in the context of the *H-2* carried by the stimulating cells. The effector cells thus produced can then react with target cells sharing with the stimulators not only minor H antigens, but also *H-2* antigens. Since the NZB strain was derived independently of other $H\text{-}2^d$ strains, such as BALB/c, B10.D2, DBA/2, HW19 (1), it very likely differs from them at a number of H loci. Furthermore, it is likely that BALB/c, B10.D2, and DBA/2 share alleles at some of these loci and differ at these same loci from NZB (22). One may, therefore, postulate that during culture of NZB cells with BALB/c stimulators, effector cells are generated against minor H antigens. However, since both strains carry the $H\text{-}2^d$ haplotype, the recognition of minor H antigens would occur in the context of the $H\text{-}2^d$ molecules. Such effectors would then be capable of lysing not only BALB/c target cells but also all targets that share with BALB/c the $H\text{-}2^d$ haplotype and at least some of the minor H antigens.

If the above explanation of the NZB anti-BALB/c reactivity is correct, then the observed reactivity is the first documented instance in which CML against minor H antigens has been obtained in primary in vitro culture. In the experiments of Bevan (23) and of Gordon et al. (24), CML to minor antigens was observed only after extensive in vivo preimmunization of the prospective donors of potential effector cells in CML. Similar requirement for in vivo pre-sensitization has also been observed in all other studies of CML against MIH antigens (25, 26). What could be the reason for the unusual behavior of the NZB cells? The one striking difference between the NZB mice and other mouse strains is the development of autoimmune disease in the NZB strain, characterized by a number of abnormalities in both humoral and cellular immunity. Prominent among these is an increased resistance to tolerance induction (9). Expanded T-cell clones specific for minor H antigens may exist in the NZB already in vivo, so that the observed reaction of NZB against *H-2* identical target cells would constitute a secondary immune reaction against these antigens. Alternatively, the NZB strain might contain factors which nonspecifically enhance the CML reaction through activation of cytotoxic T cells. Experiments to solve the question of the role of the NZB-*H-2* complex as compared to background antigens in the observed CML responses utilizing the previously mentioned B10.NZB line could not be carried out because this line has been discontinued (P. Ivanyi, personal communication, M. Micková, personal communication).

The observation that in some experiments the NZB anti-B10.D2 effectors also reacted with targets carrying haplotypes other than $H\text{-}2^d$ can be explained in one of two ways. First, it is possible that CML to some minor H antigens is not *H-2* restricted in the NZB and that the observed cross-reactivity of the NZB anti-B10.D2 cells is caused by such antigens. Second, one could argue that the cross-reactivity reflects similarity between certain seemingly unrelated *H-2* haplotypes so that T-cell effectors generated against a minor H antigen in the context of $H\text{-}2^d$ could, in the NZB, recognize this antigen in the context of $H\text{-}2^q$, for example. The reason why such cross-reactivity would be more apparent with NZB than other effectors could, again, be related to the autoimmune status of the NZB mouse. An indication that NZB effectors might be more cross-reactive than effectors derived from other mouse strains was also obtained in some allogeneic CML reactions carried out in this study. In most strain combinations CML cross-reactivity among unrelated *H-2* haplotypes is relatively difficult to demonstrate (32). In our experiments, however, cross-reactivity of NZB anti-C57BL/10 effectors on B10.D2 target cells and of NZB anti-C57BL/10 effectors on B10.D2 target cells and of NZB effectors sensitized with C57BL/6 on HW19 target cells was observed. The question of what causes the unusual CML reactivity of NZB effector cells remains open. However, by investigating this question, one may gain important information about the cellular mechanisms involved in autoimmunity.

Summary

T-cell cytotoxicity of NZB mice was tested after in vitro sensitization against a group of *H-2* identical strains (BALB/c, B10.D2, DBA/2, HW19). A highly significant and unexpected unidirectional cell-mediated lympholysis (CML) reaction by the sensitized NZB effector cells on these targets was found. After sensitization in vitro with stimulator cells of one $H\text{-}2^d$ strain, NZB effector cells ($H\text{-}2^d$) lysed all other $H\text{-}2^d$ targets and to a lesser degree, some non-$H\text{-}2^d$ targets (C57BL/10, DBA/1, B10.Q, CBA, B10.S, A.SW). NZB targets were not lysed. Differences in the major histocompatibility region between NZB and other $H\text{-}2^d$ strains could be excluded as a possible explanation for the observed reaction of NZB ($H\text{-}2^d$) against other $H\text{-}2^d$ strains. These results consequently represent the first description of a primary in vitro CML directed against determinants not coded for in the major histocompatibility complex. The responsible effector cells are demonstrated to be T cells. The CML of NZB against *H-2* identical targets appears best explained by a reaction against minor histocompatibility anti-

gens. This, and the observed cross-reactions, would indicate that the cytotoxic T-cell system in NZB mice is not subjected to restrictions found in all normal mouse strains tested until now under similar conditions. It is suggested that this hyperreactivity is related to the autoimmune responsiveness of the NZB strain.

The expert secretarial help of Ms. Monica Cassano and Ms. Jean Freeman with the preparation of this manuscript is gratefully acknowledged.

Received for publication 28 December 1977.

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KIDNEY TRANSPLANTS IN MICE

An Analysis of the Immune Status of Mice Bearing Long-Term, H-2 Incompatible Transplants*

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Transplants of living organs among mammals of the same species which differ genetically at the major histocompatibility complex are usually rejected quite promptly. There are, however, some exceptions to this which offer interesting opportunities for further study. The survival of liver transplants among certain pigs which will regularly reject skin grafts or kidney transplants from the same donors (1) is probably such an exception, but the lack of definition of histocompatibility relationships in this species limits detailed studies of the immunological mechanisms involved. Kidney transplants survive longer than skin grafts in some combinations of inbred rats, especially when their survival has been enhanced by the infusion of an antiserum specifically reactive with donor antigens (2–4). In the mouse, however, characterization of the immunogenetics of histocompatibility has been carried further than in other species so that special opportunities are offered for experiments in this species.

This report describes in detail the experimental system of kidney transplantation in the mouse. In preliminary communications we have reported that kidneys transplanted among mice involving varying degrees of histoincompatibility will regularly survive longer than skin grafts among the same strains with survival in good functioning order extending to many months in several strain combinations (5, 6). Transplant survival does not depend upon exogenous immune alterations of any kind. Prompt rejection of kidney transplants in mice can occur, however, if a sufficient immunogenetic disparity between donor and recipient is present. This is the case, for example, when C57BL/6 kidneys are transplanted to (C3H × DBA/2)F_1 recipients in which multiple incompatibilities determined by *H-2* and non-*H-2* complex genes exist. This paper will also describe the current status of our efforts, especially in one particular strain combination, to elucidate the immune relationship between long-term functioning kidney transplants and their apparently healthy, *H-2* incompatible, recipients.

Materials and Methods

Animals. Except where specified otherwise, male mice were used throughout as both donors and recipients. This selection was made because restoration of urinary drainage with our

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* Supported by grants AM-07055, CA17754, and HL-18646 from the National Institutes of Health, U. S. Public Health Service.

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technique is simpler in males. In most experiments, recipients were $(C57BL/6J \times A/J)F_1$ hybrids, hereafter referred to as $B6AF_1$, while donors were of the B10.D2N strain, referred to as B10.D2. Hybrid recipients were selected for greater size and vigor. The B10.D2 donor and $B6AF_1$ recipient combination constitutes a situation in which the genetic differences are specified at the *K* end of the *H-2* region.

In some experiments A.SW strain mice were employed as cell or tissue donors. These animals present several antigens to $B6AF_1$ recipients, including at least two private *H-2* specificities (H-2.12,19), which are not present on B10.D2 cells so that they are a suitable source of cells for ascertaining the responsiveness of $B6AF_1$ mice to foreign histocompatibility antigens other than those presented by B10.D2 donors. B10.BR mice were also used in some specificity control experiments. Cells from this strain present weaker antigenic differences to the recipient strain which are determined at the *D* end of the *H-2* region, including H-2.32. Finally, some donors of the C56BL/6 mutant strain B6.C-H(zl), abbreviated as H(zl) (7), were employed in kidney transplantation to $B6AF_1$ recipients. This donor strain was of interest as it has been reported by others (8) and confirmed in our laboratory (P. Sugarbaker and H. J. Winn, personal communication) that prompt rejection of skin grafts and active stimulation of recipient strain lymph node cells by donor cells in mixed lymphocyte culture (MLC) occurs with the incompatibilities represented in this combination. Nevertheless, no evidence of detectable humoral antibody production to donor cells, even after repeated stimulation in vivo, has been demonstrated.

Operative Procedures on Mice

SKIN GRAFTING. Full thickness skin grafts were performed according to the method of Billingham and Medawar (9). Median survival times (MST)[1] of skin grafts were calculated, where feasible, by the method of Litchfield (10) and statistical comparisons were made by Student's *t* test.

KIDNEY TRANSPLANTATION. Donor and recipient mice weighing from 25 to 30 g were anesthetized with chloral hydrate (0.1 ml of a 3.6% solution per 10 g of body weight). The left kidney of the donor was mobilized with the ureter and bladder (Fig. 1).

In the recipient the left renal artery and vein were ligated together, and the kidney was removed. After ligation of appropriate lumbar branches, ties were placed around the aorta and vena cava together caudad and individually cephalad to the intended site for the vascular anastomoses. After occlusion of the aorta and vena cava by tightening the loops, longitudinal openings were made in each.

The donor was infused systematically with 0.5 ml of a chilled balanced salt solution containing 7.5% heparin sulfate (7,500 U.S.P. U in 100 ml lactated Ringer's solution) into the vena cava. Aortic arterial inflow to the kidney was occluded and the donor tissue mass was removed including segments of aorta, vena cava, and the ureter with the dome of the bladder. End to side vascular anastomoses were constructed employing a continuous 10-0 nylon suture (Ethicon, Inc., Somerville, N.J.). The previously placed ties were removed allowing blood flow to the transplant. These maneuvers were facilitated by use of a dissecting microscope at varying magnifications for different portions of the procedure. Renal ischemia time was held to less than 35 min.

The donor ureter was next passed under the left vas deferens to reduce the chance of its kinking and the domes of the donor and recipient bladders were tailored to fit. The anastomosis of the two opened bladders was accomplished by using 9-0 nylon suture (Ethicon) in running fashion. The right kidney was then removed. Although the operation could be completed in 98% of recipients, 20–30% of animals failed to survive through the 3-day period immediately thereafter. These deaths were ascribed to the effects of operative trauma and animals were admitted to the various experiments only if they survived for 3 days after transplantation.

The status of the transplanted kidney was evaluated by several means: the continuing survival of the recipient, serial blood urea nitrogen levels (BUN) by using a standard colorimetric urease micromethod which requires about 0.02 ml of serum (blood samples being drawn from the retroorbital plexus), and by microscopic examination of sections of selected transplants. Technical restrictions did not permit serial biopsy of an individual kidney transplant.

KIDNEY SLICE IMPLANTATION. In some experiments multiple slices of renal tissue, each no

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[1] *Abbreviations used in this paper:* BUN, blood urea nitrogen; Con A, concanavalin A; KTxS, kidney recipient serum; MST, median survival time; SRBC, sheep erythrocytes.

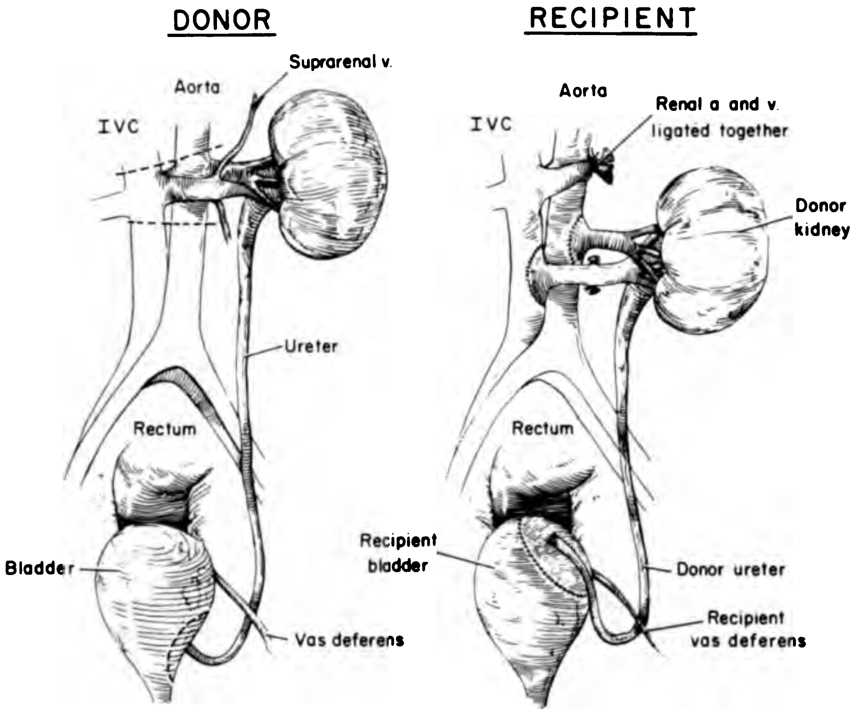

FIG. 1. Diagram of the technique used for kidney transplantation in the mouse. Note that generous portions of donor aorta and vena cava are removed as part of the donor tissue specimen. A similar approach is taken to the restoration of urinary drainage with a large patch of donor bladder. In male recipients the ureter is passed behind the vas deferens (as shown) to avoid kinking (see text).

thicker than about 0.5 mm, were implanted as free grafts into clefts prepared in the hamstring muscles of recipients. In previous experiments with endocrine tissues we have found this to be a satisfactory recipient site for tissue implants of this thickness (11).

SPLENECTOMY. Splenectomy was performed through a subcostal incision with chloral hydrate anesthesia, as described above, and standard surgical technique.

Graft Versus Host Assay. As one method of assessing the immunological capacity of recipient lymphoid cells, the cells to be tested were injected intravenously into neonatal B10.D2 mice within 12 h of birth. The weights of these animals were recorded every other day thereafter and compared to those of uninjected controls reserved in each litter. We have found this "weight gain assay" to be a useful system for demonstrating the immunological competence of murine cells in vivo by their capacity to mount a graft versus host response which is manifested by growth retardation and death of injected mice (12).

Tumor Neutralization Tests. As a further test of the immunological capacity of recipient cells to respond to donor antigens in vivo their ability to inhibit the growth of a tumor of donor *H-2* genotype was evaluated. For this purpose the DBA/2 mastocytoma P815-X2 was employed. Lymph node and spleen cells from various sources were mixed in appropriate ratios with one another and then with tumor cells before injection subcutaneously into B6AF$_1$ female recipients. The dimensions of the resultant tumors were recorded daily thereafter and were interpreted according to the original description by Winn of the "tumor neutralization test" (13). Ongoing estimates of tumor size were expressed as the average of the greatest and least diameters of a tumor.

In Vitro Studies of Lymphoid Cell Responses. Several in vitro assays of the immune responses of lymphoid cells were employed.

MIXED LYMPHOID CELL CULTURES (MLC). Mouse lymphoid cell suspensions were cultured in RPMI-1640 medium (Grand Island Biological Co., Grand Island, N.Y.) containing 5 mM Hepes buffer (Aldrich Chemical Co., Inc., Milwaukee, Wis.), 6% human serum, 100 U of penicillin G, 150 μg of L-arginine, 2 mM L-glutamine, and 75 μg of kanamycin sulfate/ml for 5 days. Cell suspensions were transferred to 12 × 75-mm disposable glass tubes and incubated at 37°C in a humidified atmosphere containing 5% CO_2. "One-way" cell proliferation was achieved by use of irradiation to block cell division in one of the cell populations. These cells were submitted to 1,000 rads from a ^{137}Cs source at a rate of 950 rads/min. Approximately 18–19 h before the cell cultures were harvested, a pulse of 0.5 μCi of [^{3}H]thymidine/ml was added (sp act 6.0 Ci/mmol; Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y.). The harvesting procedure has been reported previously (14).

^{51}CR RELEASE ASSAY. The P815-X2 tumor was utilized as the ^{51}Cr-labeled target ($Na_2{}^{51}CrO_4$; sp act, 1.0 Ci/ml; New England Nuclear Corp., Boston, Mass.) to determine the presence of killer cells directed specifically against H-2K^d antigenic determinants as is appropriate in the combination of strains mainly employed in these experiments (B10.D2 to B6AF$_1$). Concanavalin A (Con A) activated lymph node cells from A.SW mice served as targets to evaluate the specificity of killer cell induction. Blast cells recovered after a 48–72-hour incubation with 5 μg of Con A/ml were labeled in 0.5 ml phosphate-buffered saline with 100 μCi ^{51}Cr for 1 h at 37°C. The cells were then washed three times before use.

The assay as described by Brunner et al., was modified slightly (14). 1 ml each of ^{51}Cr-labeled target cells (2–5 × 10^4/ml) and of lymphoid cells were mixed and centrifuged at 225 *g* for 5 min. At the end of the culture period the cells were briefly resuspended and centrifuged at 900 *g* for 10 min. The percentage of ^{51}Cr released into the respective supernatant solutions was determined by dividing the specific experimental value of radioactive counts by the total amount of radioactivity detected in both the supernatant fluid and pellet of ^{51}Cr-labeled target cells cultured alone.

ASSESSMENT OF THE GENERATION OF KILLER CELLS IN VITRO. 1 ml each of allogeneic irradiated and nonirradiated cells, 2.0 × 10^6 cells/ml, were mixed and incubated together for 5 days. At least 10 tubes of each combination were cultured to obtain a sufficient cell yield so that various dilutions of viable recovered cells could be mixed with ^{51}Cr-labeled P815-X2 target cells in the chromium release assay (see above).

ASSESSMENT OF SERUM-MEDIATED INHIBITION OF CELLULAR RESPONSES IN VITRO. Recipient strain lymph node cells were incubated in a 1:4 dilution of either normal mouse serum or kidney recipient serum (KTxS). The cells were suspended at a concentration of 15 × 10^6/ml of the diluted serum. After a 30-min incubation in a 37°C H_2O bath, the cells were counted, resuspended, and cultured at a concentration of 2 × 10^6/ml with an equal number of irradiated donor strain cells. After 5 days in culture, the cytolytic activity of the cells was determined in a ^{51}Cr release assay. In control experiments an antiserum against H-2K^d antigens was employed. This was collected from normal B6AF$_1$ mice which had been repeatedly injected with B10.D2 spleen and lymph node cells. It had a complement-dependent cytotoxic titer of 1:512.

Assessment of Humoral Response to Sheep Erythrocytes (SRBC). Mice received intravenous injections of 0.1 ml of a 2% suspension of SRBC in saline on days 0 and 7. Blood was drawn repeatedly from individual animals to determine hemagglutination and hemolysin levels in the plasma. The techniques used in these assays were those described by Silver et al. (15).

Histology. Tissue specimens were fixed by immersion in 10% neutral formalin in 50% ethanol, cleared in butanol, cut at 4 μm after embedding in paraffin, and stained with hematoxylin and eosin.

Cytotoxic Antibody to Cells. Cytotoxic antibody levels were determined in vitro against donor strain spleen or lymph node cells in a two stage test. Rabbit serum was used as the source of complement and trypan blue dye exclusion as the criterion of cell viability as described previously (16).

Results

Renal Isotransplants. Bilateral nephrectomy and renal isotransplantation from B6AF$_1$ donors was performed in 10 B6AF$_1$ mice. Three animals died on the 2nd day and were therefore not considered further in the study. One of the remaining seven recipients still survives at 876 days (Fig. 2). One animal died on day 51 and five were sacrificed on days 48, 499, 512, 516, and 656 days. The

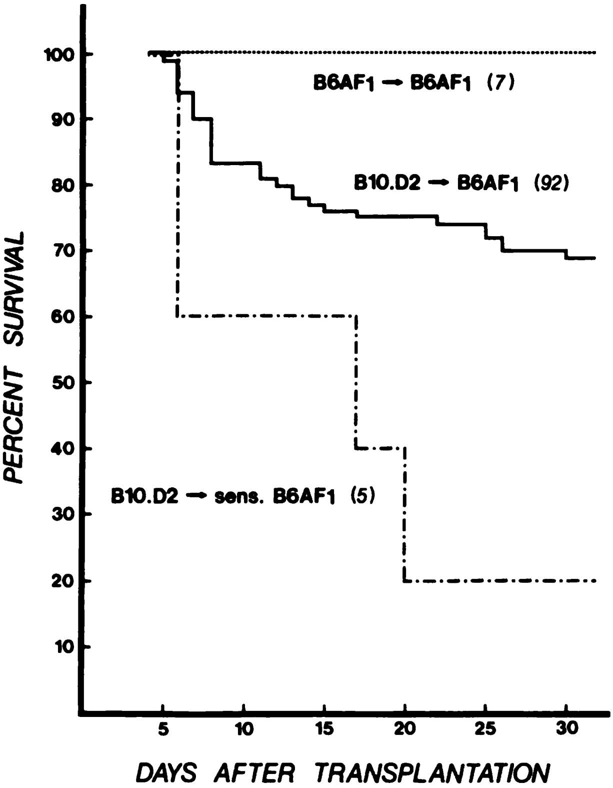

FIG. 2. The survival of renal isotransplants (B6AF$_1$ to B6AF$_1$) is compared to that of allotransplants from B10.D2 donors to normal B6AF$_1$ recipients or to B6AF$_1$ recipients which have been previously immunized to B10.D2 cells (see text). The number of animals in each group is indicated. Only those recipients which survived for at least 3 days are included in these data. About 30% of the transplants to normal recipients fail during the 1st mo after transplantation. Very few are lost thereafter. A much higher percentage of transplants to preimmunized recipients is destroyed early.

BUN levels of the seven survivors, measured between 30 and 45 days after transplantation, were 20, 22, 23, 24, 26, 27, and 40 mg/100 ml (average 26.0 mg/100 ml). On examination of sections of fixed tissue from these transplants little evidence of inflammation was seen and signs of significant ureteral obstruction were generally minimal.

In other control experiments it was found that eight animals undergoing unilateral nephrectomy survived indefinitely, maintaining BUN levels in the normal range, i.e., 15–30 mg/100 ml with an average of 22.4 mg/100 ml.

Bilaterally nephrectomized B6AF$_1$ mice succumbed to uremia within 36 h with BUN levels from 80 to 200 mg/100 ml.

Survival of Allogeneic Kidney Tissue Transferred to Normal Recipients

KIDNEY TRANSPLANTS TO NORMAL RECIPIENTS. B10.D2 kidneys were transplanted to normal B6AF$_1$ male mice and the course of the animals was followed. The pattern of survival of these animals is depicted graphically in Fig. 2. Since

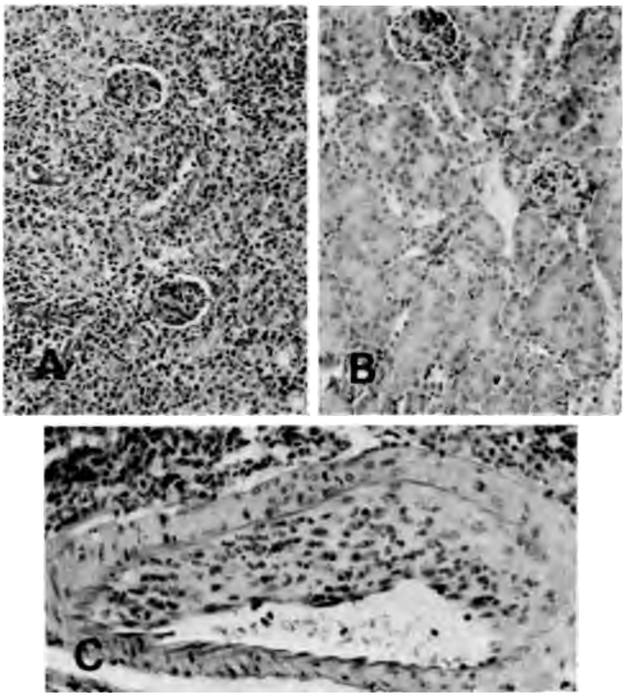

FIG. 3. Histological changes observed in B10.D2 kidneys transplanted to $B6AF_1$ recipients. (A) Allotransplant removed at 9 days after transplantation. An intense mononuclear infiltrate is present and is associated with tubular damage and margination along arterial endothelium. This and other sections are stained with hematoxylin and eosin (no. 338, × 190). (B) Allotransplant removed at 56 days after transplantation. No infiltrate is present and the tubules appear normal. The top glomerulus has a segmental scar (no. 697, × 190). (C) Allotransplant removed at 71 days after transplantation. This large artery shows marked intimal proliferation and infiltration by mononuclear cells. Only a scant interstitial infiltrate is present in the cortex and the smaller arteries are normal (no. 687, × 300).

early survival of kidney isotransplants exceeds that of allotransplants, the loss of about 30% of the allotransplants in the 1st mo must be attributable mainly to rejection. By following the course of 92 recipients of allografts, it was apparent that deaths became much less common after the 1st mo. Late kidney failure may, of course, be due to the cumulative consequences of technical factors as well as rejection activity. During the 70-day period from 30 to 100 days postoperatively, the additional loss of animals with gradually advancing uremia was about 20%. Several recipients have survived for as long as 1 yr with normal renal function. Mice that survive longer than this tend to have a gradual rise in their BUN levels.

Early rejection is characterized by intense mononuclear infiltration. Mononuclear cells invade tubules, accumulate along the arterial endothelium, and may mediate tissue damage at these sites (Fig. 3A). An adaptive phase occurs subsequently as time progresses. Samples taken during this period showed a remarkable regression of the interstitial infiltrate and little or no evidence of residual tubular damage (Fig. 3B). A late phase follows in which some tissue damage, especially to arterial vessels and glomeruli, gradually appears. The changes consist of some mononuclear cell infiltration and intimal proliferation beginning in larger renal arteries (Fig. 3C). Similar lesions are well known features of human renal allografts (17). Glomerular damage seems to be slowly progressive over the duration of survival of the allografts. The mechanisms for these late changes have not yet been established.

Seven B10.D2 kidneys from female donors were transplanted into female $B6AF_1$ recipients to explore the possibility of a difference in responsiveness of female recipients to transplanted kidneys in this strain combination. Five of these survived in excellent condition for over 4 wk, thus demonstrating no detectable difference attributable to the sex of the recipient.

12 H(zl) kidneys transplanted to $B6AF_1$ recipients were studied. Of these, 9 survived beyond 3 days in excellent condition with BUN levels ranging from 19 to 36 mg/100 ml for at least 5 wk. Accordingly, their survival was quite similar to that seen for B10.D2 kidneys to $B6AF_1$ recipients.

KIDNEY IMPLANTS. Two to four B10.D2 renal slices comprising, in aggregate, at least one third of a kidney were implanted bilaterally into the hamstring muscles of each of 12 adult male $B6AF_1$ recipients. Donor kidneys were perfused exactly as for transplantation before preparing the tissue slices to eliminate any variation in number of "passenger leukocytes." In selected cases blocks of muscle tissue containing the implants were excised 11–14 days after grafting for histological examination. By this time the normal renal architecture of the implants was scarcely recognizable as it was largely effaced by a dense, mononuclear cell infiltrate which was considered to be entirely characteristic of allograft rejection. Donor strain skin grafts were applied later to some of these recipients (see below).

Kidney Transplants to Preimmunized Recipients. Five male $B6AF_1$ mice, which had received and rejected B10.D2 skin grafts, underwent bilateral nephrectomy and transplantation of B10.D2 kidneys from 1 to 14 days after skin graft rejection was complete. Their survival is shown in Fig. 2. Early loss of transplants was much greater in this group. Indeed, over half were unable to support the life of their recipients for more than 5 days. Nevertheless, it is of interest that one animal survived for a prolonged period (122 days) before being sacrificed.

Kidney Transplants to Splenectomized Recipients. B10.D2 kidneys were transplanted to nine recipients which had undergone splenectomy from 12 to 27 days previously. Several reports have appeared (18–20) that splenectomy of a recipient impairs active immunological enhancement of transplants, although this is not agreed to by all authors (21). Accordingly, it was noteworthy that five of these kidney transplants survived in excellent condition for many weeks, thus demonstrating no influence of prior splenectomy on renal transplant survival in this group of animals.

Survival of Test Skin Grafts to Kidney Recipients. 15 $B6AF_1$ recipients were selected which had born B10.D2 kidney transplants for 40–60 days. Skin grafts from B10.D2 donors to these recipients survived as recorded in Fig. 4. The presence of a surviving kidney transplant greatly extended the life of a *later* skin graft from the donor strain with over 65% of grafts surviving for more than 1 mo. Beyond this period many grafts gradually lose their hair, shrink in area,

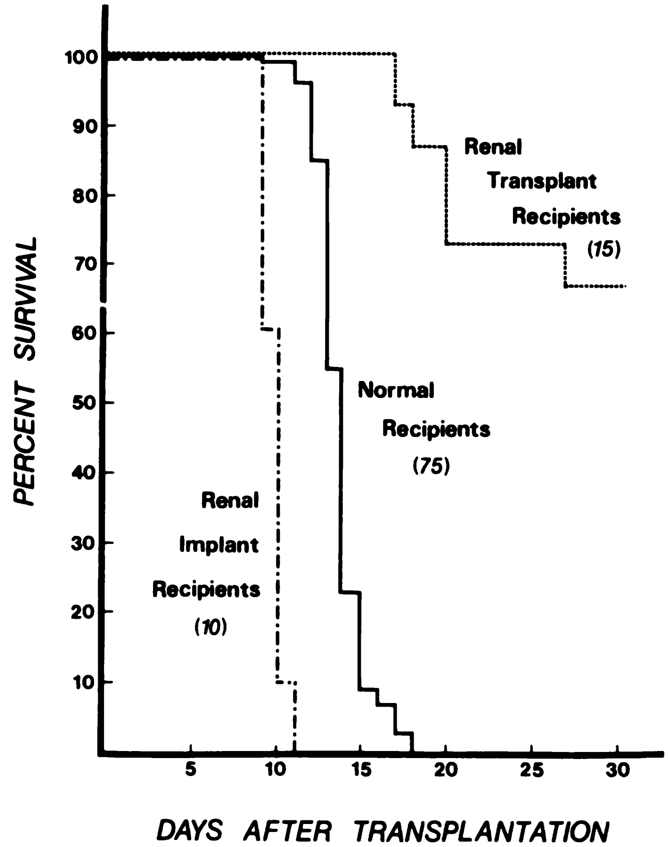

FIG. 4. The survival times of donor strain (B10.D2) skin grafts to normal $B6AF_1$ recipients or to recipients of previous grafts of B10.D2 kidney tissue. Kidney tissue was transferred either by transplantation or in free implants of renal slices to the leg muscles. The number of animals in each group is indicated. Transplant recipients, which are healthy and maintaining BUN levels in the normal range, support the prolonged survival of donor strain skin grafts whereas recipients of kidney tissue implants reject grafts in accelerated fashion. This alteration is specific for the donor strain (see text).

and are slowly rejected in succeeding weeks. In several instances, however, the grafts remained in excellent condition for well over 1 yr. In no case was there any evidence that the presence of the skin graft influenced the function of the kidney transplant. This behavior of skin grafts differed markedly from that observed with normal $B6AF_1$ recipients (MST 13.5 ± 1.1 days).

That this alteration in recipient responsiveness was particularly evident toward the donor strain is shown by the fact that skin grafts from A.SW donors to nine $B6AF_1$ kidney transplant recipients during the same period after transplantation were rejected with an MST of 10.2 ± 1.03 days (MST of A.SW skin grafts to normal $B6AF_1$ recipients is 10.5 ± 1.1 days). Another test of the specificity of immunological reactivity of kidney transplant recipients consisted of measuring their responses to SRBC. Four $B6AF_1$ recipients of B10.D2 kidney transplants which were in good condition from 13 to 166 days after transplantation were tested. Their responses to SRBC immunization, as compared to the responses of eight normal controls, were unimpaired. For example, at 21 days after SRBC, the transplant recipients showed hemagglutinin titers ranging from 160 to 640 which is the same range as that found in normal animals. This is further evidence that transplant recipients are capable of mounting normal immune responses to antigens not present in the donor tissue.

Of interest was the additional finding that B10.D2 skin grafts to 10 $B6AF_1$ mice about 4 wk after receiving B10.D2 kidney implants were rejected in accelerated fashion (MST 9.1 ± 1.02 days, Fig. 4). Accordingly, a striking difference in immune responsiveness to donor antigens occurred depending upon whether the donor kidney tissue was implanted as a free graft or was transplanted with primary vascular union.

Analysis of Immune Responsiveness of Kidney Transplant Recipient Lymphoid Cells. Several tests of the immune responsiveness of lymph node and spleen cells from $B6AF_1$ recipients bearing B10.D2 kidney transplants for 1 mo or more were made, both in vivo and in vitro.

In Vivo Tests

CELL TRANSFER TO NORMAL ADULT MICE. Lymph node cells from 11 long-term $B6AF_1$ recipients of B10.D2 kidney transplants were pooled and transferred i.p. to normal $B6AF_1$ mice in a dosage of 2×10^8 cells per mouse. This cell dosage was selected as it had been shown by Kilshaw et al. to demonstrate marked suppressor cell activity in mice in which specific unresponsiveness to allogeneic skin grafts had been induced by treatment with donor strain lymphoid cells, anti-lymphocyte serum, and *Bordetella pertussis* (22). Three animals were treated in this particular experiment with the prescribed number of cells. 2 days after receipt of the cells, B10.D2 skin grafts were placed on these animals. These grafts were rejected in 12, 14, and 15 days (MST 13.0 ± 1.03 days). The MST of B10.D2 skin grafts to 4 $B6AF_1$ mice which had received the same number of cells from mice which had recently received and rejected B10.D2 skin grafts was 10.0 ± 1.02 days. These figures were compared also with the MST of B10.D2 skin grafts to normal $B6AF_1$ recipients of 13.5 ± 1.1 days. All these skin grafts were performed at the same time as we have found that slight differences in skin graft survival can be observed from time to time even under seemingly standardized conditions. The survival of skin grafts to animals which had received cells from kidney transplant recipients did not differ statistically from the normal MST ($P > 0.05$) whereas significant acceleration of rejection was demonstrated after transfer of immunized cells ($P < 0.01$). In other experiments, done at several different times, pooled lymph node and spleen cells from a total of 17 kidney transplant recipients were transferred to normal $B6AF_1$ mice at the dosage mentioned above. No evidence of suppressor cell activity was apparent by observing skin grafts to 10 $B6AF_1$ recipients treated in this way.

Larger numbers of cells were not employed as this experimental design required an uneconomical use of cells from the necessarily small number of kidney transplant recipients available. Further tests for the presence of suppressor cells accordingly made use of other types of cell transfer systems and of in vitro tests in which fewer cells could be employed (see below).

INJECTION OF LYMPHOID CELLS INTO NEWBORN MICE. The potential for cells

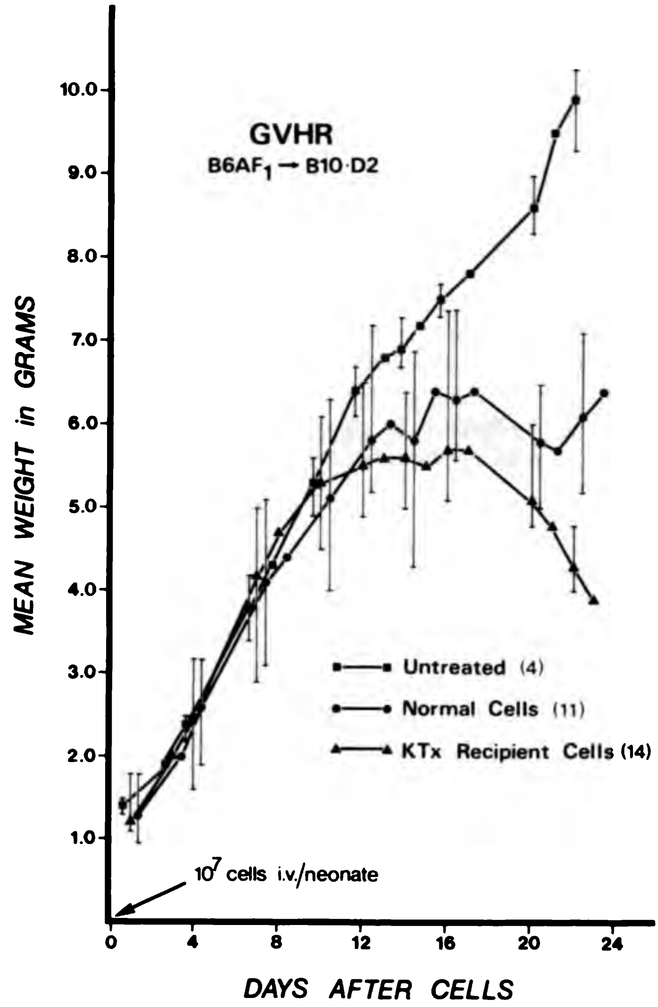

FIG. 5. The capacity of pooled spleen and lymph node cells to produce a graft versus host reaction (GVHR) (runt disease) was determined by following the gain in body weight of neonatal B10.D2 mice injected intravenously with the cells to be tested. Each of several litters was divided into three groups, some receiving 10^7 viable spleen and lymph node cells from normal adult $B6AF_1$ donors, some the same number of cells from $B6AF_1$ recipients of B10.D2 kidneys, and some receiving no treatment. The mean weights of each group are plotted. At the cell dosage tested, no difference is detected by this test between the responsiveness of cells from normal and kidney transplant recipient animals. The number of animals entering each group is indicated, and the range of recorded weights is plotted at representative points.

from long-term kidney transplant recipients to generate a specific immune response to donor strain antigens in vivo was tested by delivering 1×10^7 pooled lymph node and spleen cells intravenously to newborn B10.D2 mice and observing their gain in body weight thereafter. The results from four separate litters were combined. In each litter some neonatal mice received normal $B6AF_1$ cells, some received kidney transplant recipient cells and some were left uninjected. As Fig. 5 shows, these cells were fully capable of mounting a graft-versus-host response in neonatal hosts as they produced a retardation in growth which was indistinguishable from that after the injection of the same number of cells from normal adult B6AF$_1$ mice. Littermate controls which were uninjected developed at the normal rate.

CELL RESPONSIVENESS AS EVALUATED BY THE "TUMOR NEUTRALIZATION TEST." For these experiments the target employed was the P815-X2 mastocytoma. 5,000,000 viable tumor cells injected subcutaneously into each B6AF$_1$ recipient produced a tumor mass which reached a size of about 7–10 mm in greatest diameter by 6–8 days before the tumor cells were gradually rejected by the active immune responses of the host. When this number of tumor cells was mixed with an equal number of pooled lymph node and spleen cells from preimmunized B6AF$_1$ mice, the combined inoculum of tumor cells and immunized lymphoid cells resulted in a barely palpable growth. Lymph node and spleen cells from kidney transplant recipients mixed in equal numbers with tumor cells had no detectable influence on tumor growth in normal B6AF$_1$ mice.

A main objective in using this system was to determine whether cells from kidney transplant recipients would suppress the action of preimmunized cells on the tumor in vivo as compared to normal recipient strain cells. In these experiments inhibition of growth of the tumor caused by an equal number of sensitized cells was reduced after the addition of a like number of either normal or kidney transplant recipient cells, but kidney transplant recipient cells did not have a greater influence in this regard. Indeed, it seems likely that the effect of secondarily added cells from either of these sources was simply to dilute the impact of the sensitized cells on the tumor.

In summary, these experiments yielded no evidence that cells from long-term kidney transplant recipients can suppress the specific immune reactivity of previously immunized lymphoid cells against donor antigens in vivo under the conditions employed.

In Vitro Tests

CELL-MEDIATED CYTOTOXICITY. Spleen cells from 16 kidney transplant recipients were evaluated individually to determine whether immune cytotoxic killer cells could be detected at times ranging from 42 to 372 days after transplantation. The average quantity of ^{51}Cr released from labeled target cells did not differ significantly ($P > 0.05$) from that produced in cultures with cells from normal B6AF$_1$ mice. Assays were performed under various conditions including rocking versus stationary cultures, 4, 6, 9, or 16 h of incubation, and test to target cell ratios of 50:1, 100:1, or 200:1 (detailed data not shown). Mice which had recently rejected B10.D2 skin grafts or had received a series of intraperitoneal injections of B10.D2 lymphoid cells yielded spleens which contained easily detectable killer cell activity.

Experiments in which test cells were mixed in varying proportions with target cells indicated that few if any cytotoxic killer cells are present in the spleens of kidney transplant recipients at 40 days or longer after transplantation. Similar results followed when lymph node cells were tested in this way (Fig. 6).

MIXED LYMPHOCYTE CULTURES. The proliferative responses of lymphoid cells from kidney transplant recipients after stimulation with irradiated donor

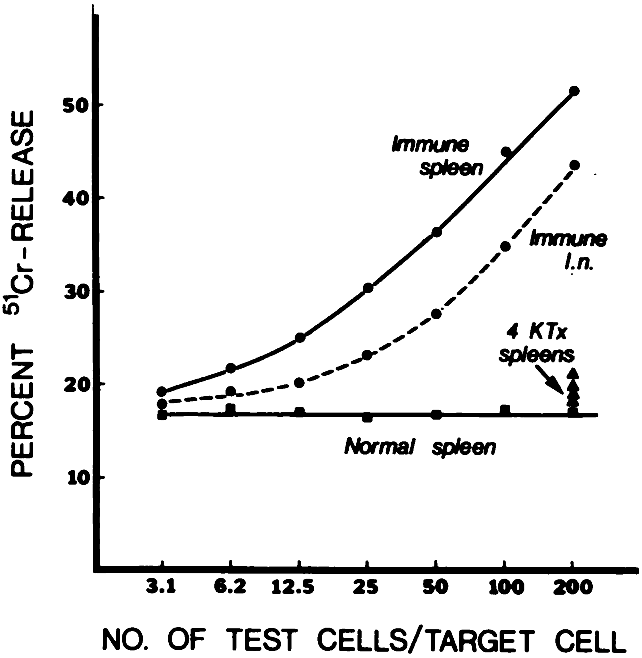

FIG. 6. A comparison of the ability of lymphoid cells from kidney transplanted, immune and normal animals to cause killing in vitro. Specifically immune lymphoid cells were obtained from B6AF$_1$ mice which had been grafted on two consecutive occasions with skin from B10.D2 donors (days 0 and 19), and which were then injected on day 29 with 7 × 10^7 B10.D2 lymphoid cells. Spleens and lymph nodes were removed 4 days later and titrated for killer cell activity in a ^{51}Cr release assay against P815-X2 tumor cells. The kidney transplant recipients tested had received B10.D2 kidneys 54, 56, 59, and 63 days before removal of their spleens and lymph nodes (l.n.). Lymphoid cells from normal B6AF$_1$ mice served as controls. Supernates from the assays were evaluated for ^{51}Cr-release after an 8 h incubation. Spontaneous ^{51}Cr-release was 19.8%. Active killer cells were not detected in the spleens of kidney transplant recipients as compared to the killing activity of different numbers of specifically immune cells.

strain cells were compared to the levels of response attained by normal recipient strain cells. The responses of three long-term survivors of kidney transplants (118–199 days) were similar to normal levels of response (Table I).

GENERATION OF KILLER CELLS. Kidney transplant recipient spleen cells from these same mice were also incubated with irradiated donor strain cells to determine their ability to generate cytotoxic effector cells. While significant cytolysis was detected in the subsequent CMC assay, the levels of killing attained were substantially lower than the levels of killer cell activity generated by cells from normal B6AF$_1$ mice (Fig. 7). Further studies have confirmed these results in that nucleated splenic cells from five out of six mice bearing foreign kidney transplants for 118 days or longer produced lower levels of killer T cells than cells from normal mice.

In another series of experiments, spleen cells from kidney transplant recipients were sensitized in vitro against irradiated B10.D2 and A.SW cells. While only one out of five recipients yielded detectable levels of killer cells against the

TABLE I
Responses in MLC of KTxR and Normal $B6AF_1$ Cells

Kidney transplant recipients*					Normal $B6AF_1$		
Animal no.	Days post Tx	Cell source	Alone	+B10.D2 (1,000 rads)	Cell source	Alone	+B10.D2 (1,000 rads)
			cpm			*cpm*	
529	118	LN	1,677	53,145	LN	11,744	148,588
		Spleen	22,937	108,145	Spleen	25,195	97,149
507	199	LN	3,028	113,216	LN	729	82,756
535	134	LN	15,488	49,818	LN	2,737	37,606

* B10.D2 into $B6AF_1$ kidney transplants.

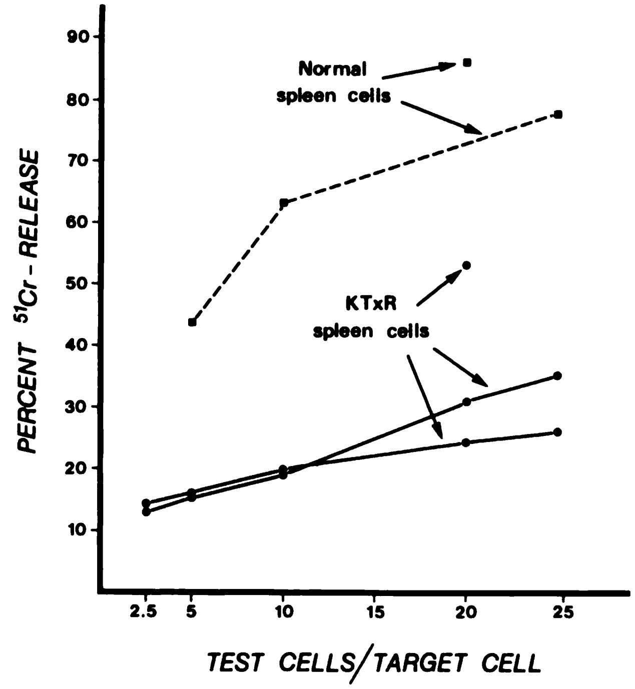

FIG. 7. The ability of $B6AF_1$ lymphoid cells from long-term recipients of B10.D2 kidney transplants to become sensitized in vitro. Nucleated splenic cells from transplant recipients and normal $B6AF_1$ mice were cultured with irradiated donor strain cells (B10.D2) at 2×10^6 each/culture. 5 days later the cultured cells were evaluated in a ^{51}Cr-release assay to measure the level of sensitization that had developed. Spleen cells from kidney transplant recipients show reduced sensitization to donor antigen by this test as compared to cells from normal mice.

donor strain H-2^d targets, five out of six developed significant levels of killer activity directed against the A.SW H-2^s target antigens (data not shown). This is further evidence that transplant recipients have specifically depressed abilities to generate killer cells against the antigens of the donor kidney.

SUPPRESSOR CELL ASSAYS. Two types of tests were employed. One was designed to reveal a suppressive effect on the generation of effector cells from

TABLE II

Effect of Preincubated Kidney Transplant Recipient Cells (KTxR) on Killing Activity of Immune Cells

Preincubated mixtures added to CMC assay‡	% Specific ^{51}Cr-release* Cell donors for CMC assay		
	None	Nonimmune B6AF$_1$	Immune B6AF$_1$
None	–	1.8	55.4
Normal cells alone	0	1.2	56.0
KTxR cells alone§	0	1.2	56.0
Normal cells + P815-X2‖	0	0.3	46.7
KTxR cells + P815-X2	0	0	44.0
Normal cells + B10.D2 (1,000 rads)¶	0	1.6	53.1
KTxR cells + B10.D2 (1,000 rads)	0	1.6	50.9

* Spontaneous release = 33.0% after 17 h CMC assay.

‡ Preincubated at 37°C for 23 h and used in CMC assay at a ratio of 1:1 with nonimmune and immune B6AF$_1$ cells.

§ Pool of spleen cells from two kidney transplant recipients.

‖ Normal and KTxR cells preincubated with P815-X2 at 100:1.

¶ Normal and KTxR cells preincubated with B10.D2 cells at 3.3:1.

normal cells and the other to evaluate a suppressive effect on the expression of immunity by preimmunized cells.

For the first test kidney recipient cells and normal B6AF$_1$ cells were mixed at ratios of 1:6, 1:3, and 1:1 and were sensitized together in culture for 5 days by irradiated donor strain stimulating cells. No suppression of the normal response was detectable at any ratio of kidney recipient cells to normal cells when these cultured cells were tested in a CMC assay.

In the second test for suppression, normal or kidney recipient spleen cells were incubated for 23 h with tumor target and irradiated donor strain cells. The effect of these preincubated cell mixtures on the capacity of immune cells to kill ^{51}Cr-labeled tumor targets was assessed. No suppression of immune effector cell function was evident in any of the combinations (Table II). Similar procedures have been reported to reveal evidence of suppressor cells in NZB mice against syngeneic or autochthonous fibroblasts (23). The lower levels of ^{51}Cr-release in the assays with mixtures preincubated with P815-X2 tumor cells probably reflects the slight inhibition which is to be expected from competition by unlabeled target cells. No killer cells were generated by cells from either kidney transplant recipients or normal B6AF$_1$ animals during the 23 h of incubation with either irradiated B10.D2 cells or P815-X2 tumor cells.

These data indicate that kidney transplant recipients contain approximately normal numbers of lymphoid cells that proliferate in response to H-2 antigenic stimuli (helper T cells) but that long-term recipient mice are deficient in their functional prekiller T-cell subpopulations. Also, despite the presence of the transplant, the cell responses attained in vitro are substantially lower than would be expected for secondary responses indicating again that these recipients have few or no immune "memory" cells.

Analysis of Serum from Kidney Transplant Recipients. Serum was collected repeatedly from the retro-orbital sinus of 16 B6AF$_1$ recipients of B10.D2 kidney transplants at times ranging from 30 to 300 days after transplantation. The tests to be described were performed with a single pool of serum from these collections. Complement-dependent cytotoxic antibody was not detectable by our technique in this serum pool. Individual serum samples from transplant recipients, however, were sometimes shown to contain cytotoxic antibody (see below).

IN VIVO TESTS. To test the capacity of this serum to enhance the survival of donor strain test skin grafts, a regimen for serum administration was employed which exceeded in dosage (volume) that previously shown in our laboratory to be effective in producing enhancement of skin grafts in this strain combination with several antisera which contained demonstrable cytotoxic activity (24). 10 mice received 0.2 ml of serum i.p. on days 0, 2, and 4 with respect to receiving B10.D2 skin grafts. The MST of these grafts was 14.0 ± 1.1 days. When compared to the MST of such grafts on untreated recipients (13.5 ± 1.1 days) the difference was not significant ($P > .05$).

IN VITRO TESTS. (*a*) Cytotoxic antibody titers were determined repeatedly on 46 B6AF$_1$ recipients of B10.D2 kidneys. The percentage of animals with detectable antibody titers declined from 24% at 4 wk to 7% after 12 wk. Antibody titers also declined from an average of 1:64 at 4 wk to 1:4 after 12 wk.

(*b*) The recipient serum pool was tested to determine whether it could inhibit the cytolytic activity of immune T cells and/or the ability of normal B6AF$_1$ cells to generate effector T cells during sensitization in vitro.

Kidney recipient serum and normal mouse serum, each at a concentration of 1:10, were added to tubes containing decreasing concentrations of sensitized B6AF$_1$ cells. ^{51}Cr-labeled P815-X2 target cells were also added and the mixtures incubated for 4.5 h as outlined in Materials and Methods. No inhibition of killing activity was effected by the kidney recipients' serum (Table III). The inability of this serum to block killing activity of sensitized cells is particularly evident at lower ratios of sensitized to tumor target cells. Antiserum raised in B6AF$_1$ mice against B10.D2 skin and/or lymphoid tissues blocked killing in this assay completely (data not shown).

The same kidney recipient serum pool could, however, suppress the generation of killer cells in cultures in which normal B6AF$_1$ cells were mixed with irradiated B10.D2 donor strain cells. Serum from long-term recipients of B10.BR strain kidneys also depressed the ability of B6AF$_1$ cells to generate killer cells against H-2K^d antigenic targets (see reference 6 for results with this strain combination). The suppressive effect observed with the kidney recipients' serum, therefore, does not appear to be antigen specific. Its influence, however, is confined to a limited "phase" of the development of the immune response in that the serum does not suppress the activity of immune cells but depresses the ability of nonimmune cells to differentiate into effector cells. The nature of this suppressive substance offers an opportunity for further investigation.

TABLE III
Effect of Kidney Transplant Recipient Serum on the Killing Activity of Immune Cells

Type of serum	% ^{51}Cr-release* Sensitized cells/Target cell‡			
	20:1	10:1	5:1	2.5:1
NMS	78.3	70.2	54.2	45.6
KTxR Serum	76.9	71.5	61.6	46.0

* Spontaneous release of ^{51}Cr-labeled P815-X2 = 22.9% after a 4.5-h assay.

‡ B6AF$_1$ cells were sensitized for 5 days in vitro against irradiated B10.D2 cells.

Discussion

These experiments have demonstrated that transplant rejection in several combinations of mouse strains is much less vigorous against kidneys than against skin grafts from the same donor strain. That this difference does not depend upon antigens peculiar to skin was shown by the fact that slices of renal tissue, implanted as free grafts, were rejected at about the same rate as skin grafts. The recipients of implants of renal tissue are specifically immunized after graft rejection. By contrast, however, primarily vascularized renal transplants provoke a much weaker rejection response which tends to diminish with time. This is suggested by the distinct clearing of infiltrating leukocytes from transplanted kidneys after the first 3–4 wk and by the diminished prevalence of complement-dependent cytotoxic antibody in the sera of transplant recipients as time progressed.

In another series of experiments employing primarily vascularized heart transplants between mice, Corry et al., in our laboratory, made some similar observations with B10.BR hearts transplanted to B6AF$_1$ recipients. In this strain combination, the transplanted hearts functioned well for about 3 wk but then entered a period during which the heart beat was greatly reduced before it was restored spontaneously to normal (25). In a later study, Corry found that donor strain skin grafts were rejected in somewhat accelerated fashion in this strain combination if applied soon after heart transplantation but that later skin grafts enjoyed prolonged survival (26). It appears that although heart transplants have the capacity, under the right conditions, to induce an alteration in recipient immune responses, they are less able to do so than are kidney transplants. This is certainly the case in the strain combination we have studied most intensively (B10.D2 donors and B6AF$_1$ recipients) since heart transplants are all promptly rejected in contrast to the long survival of kidneys transplanted between these strains.

The set of circumstances which evolves spontaneously in our animals may be similar to that obtaining with long-surviving kidney or heart transplants in some rats which can be produced by the treatment of recipients with a short course of injections of an antiserum directed against the foreign antigens of the donor. Generally, F$_1$ transplants to a parental strain recipient are required for long-term survival. Evidence has been advanced, especially by Batchelor and his colleagues (27) that these animals enter a "steady state" which persists long after the originally infused antibody has disappeared. In our untreated recipients it is unclear as to whether the antibody against donor cells which was often detectable contributed to the development of the steady state which developed in them. It is probable that antibody at low levels is present throughout the survival of the recipient. Nevertheless, two facts from our in vivo experiments make it unlikely that antibody is indispensable to the suppression of the early rejection reaction. First, the fact that prior splenectomy, which generally lessens the vigor of active enhancement, did not curtail the survival of transplanted kidneys does not support the concept that conventionally defined enhancement contributes to the onset of the "steady state" in our animals. Secondly, the fact that kidneys from H(zl) donors survived in the absence of any detectable antibody to them is evidence in the same direction.

The immune alteration in recipient responsiveness after the transplantation of a kidney in the strain combinations tested has a considerable degree of specificity. Donor skin grafts (B10.D2) often survived for many weeks whereas skin grafts presenting strong new antigens (A.SW) were rejected normally. The response to sheep erythrocytes was also normal. Nevertheless, there is no justification for classifying this phenomenon as an example of actively acquired immunological tolerance as there is ample evidence that lymphoid cells from kidney transplant recipients can mount vigorous immune responses to donor antigens both in vivo and in vitro. Furthermore, no evidence could be found for the participation of suppressor cell activity by either in vivo or in vitro tests.

Although graft versus host assays established that cell populations from kidney recipients were capable of producing effective immune responses in vivo which were indistinguishable from those generated by cells from normal donors, the cell doses used were large and the discriminatory capacity of the test employed was probably insufficient to reveal reductions in immune responsiveness of the inoculum. In vitro tests offer easy flexibility in varying the numbers of cells in the reaction mixture and these provided evidence for a reduction in cellular responsiveness. It must be remembered that this balanced system probably involves the continual turnover of potentially reactive cells which are constantly being influenced by their surroundings, and single samplings of the components of such a system may not reveal how the balance is maintained. Nevertheless, it was considered significant that long-term recipients, on repeated testing, showed a reduction in functional precursors of killer T cells as well as showing no evidence for the presence of active killer T cells themselves, whereas such cells were readily produced on stimulation by antigens which had not been present in the transplanted kidney. This deficiency of immune cells and in pre-killer cells appears to be specific in that relatively normal levels of killer cells could be induced against A.SW target cells. The fact that almost all transplanted kidneys were rejected promptly when transplanted to recipients which had previously been immunized by donor strain skin grafts shows that such rejection is possible and is consistent with the notion that immune effector cell generation is somehow insufficient to produce decisive rejection by normal recipients.

The nature and significance of the suppressive substance detected in recipient serum by in vitro tests have not been established. Further studies to define the mechanism of this specific deficiency are needed. Meanwhile, an additional dimension of understanding of the immunological balance between the transplanted organ and its surviving host has been gained by various approaches to upsetting this balance as reported in an accompanying paper (28).

Summary

Kidney transplants between strains of mice which are incompatible at either the *K* or the *D* end of the *H-2* complex usually function for prolonged periods supporting the lives of nephrectomized recipients. This occurs with no recipient treatment. With multiple *H-2* and non-*H-2* determined incompatibilities, transplants may be rejected but more slowly than skin grafts. In the strain combination studied most extensively in these experiments (B10.D2 to $B6AF_1$) in which the incompatibility was confined to the *K* end of the *H-2* region, about 70% of recipients survived for many weeks with normal blood urea nitrogen levels. Skin grafts between untreated members of these strains were rejected promptly (mean survival time of 13.5 ± 1.1 days) as were kidney transplants to recipients of prior skin grafts. Donor strain skin grafts to recipients of kidney transplants after kidney transplantation enjoyed greatly prolonged survival whereas skin grafts from a third party (A.SW) were rejected normally. If kidney tissue was transferred in the form of free grafts without primary vascular union, it was rejected promptly leaving its recipient highly immunized.

Cellular and humoral immunity to donor antigens declined over the first few weeks after transplantation, and the spleens of long-term recipients contained no "killer cells." Recipient lymphoid cells could mount active graft versus host reactions to donor strain antigens on transfer to neonatal mice. Nevertheless, they were distinctly less able to respond specifically by the production of killer cells to donor strain antigens after sensitization in vitro. No evidence that this defect was associated with the presence of suppressor cells was forthcoming from several types of in vivo and in vitro tests.

We would like to acknowledge the effective aid of Ms. Susan W. Ayres in the preparation of the manuscript and the helpful comments of Dr. Henry J. Winn.

Received for publication 29 December 1977.

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INDUCED IMMUNE DESTRUCTION OF LONG-SURVIVING, H-2 INCOMPATIBLE KIDNEY TRANSPLANTS IN MICE*

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We have reported in an accompanying paper that kidney transplants to mice regularly produce an immune alteration toward donor antigens without any other treatment (1). This immune alteration takes place over the 1st mo or so after the recipient has undergone kidney transplantation and bilateral nephrectomy. In several strain combinations in which incompatibility determined by genes of the *H-2* system is involved, manifestations of rejection activity directed toward the transplant tend to diminish during the early weeks after transplantation and loss of transplanted kidneys becomes much less common.

The mechanism by which this equilibrium between transplant and recipient is maintained remains to be established in full. In our previous paper we presented evidence that the lymphoid cells of kidney transplant recipients which have survived 1 mo or more are able to respond to donor antigens both in vivo and in vitro, but that they do so to a lesser degree, as a population, than do normal cells. This relative reduction in ability to respond is associated with the presence in the recipients' sera of a substance which interferes with the full development or expression of an immune response, especially the development of killer T cells.

The present experiments made use of mice with long-term, well functioning kidney transplants from *H-2* incompatible donors. These animals offer a study system in which various methods of inducing the immune destruction of the surviving organ can be tested. Each recipient bears a large mass of foreign tissue which presents well-characterized antigenic differences to its host. The function of the allogeneic organ can be evaluated on a continuing basis by measuring the blood urea nitrogen level of its recipient. The experiments described here include treatments in which heightened reactivity to donor tissue antigens was conferred by passive immunization and also maneuvers which awakened the dormant responses of the host so that the recipients responded more strongly to donor antigens resulting in the acute destruction of the transplant.

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* Supported by grants AM-07055 and HL-18646 from the National Institutes of Health, U. S. Public Health Service. Some of the results embodied in this paper were presented in a lecture to the British Society of Immunologists and the British Transplantation Society on 21 October 1976.

Materials and Methods

Animals. Inbred mice were used throughout. As described previously (1) strain combinations in which incompatibilities were restricted to a portion of the *H-2* region were employed. The principal test system involved B10.D2 mice as donors and (C57BL/6 × A/J)F_1, abbreviated B6AF_1, mice as recipients. This strain combination involves an incompatibility determined at the *K* end of the *H-2* complex (H-2.31) as well as an incompatibility in the *Ia* region. Cells from A.SW mice were also used in some experiments as they present different *H-2* incompatibilities (H-2.7, 12, 19, 51) to B6AF_1 recipients from those offered by B10.D2 cells.

Members of the B10.BR strain were also used in one experiment as a source of both kidneys and lymphoid cells for transplantation to B6AF_1 recipients. This donor strain is incompatible with the recipient strain at the *D* end of the *H-2* complex (*H-2D* plus *I-C*), the principal antigenic difference being H-2.32. Members of this strain are less incompatible with B6AF_1 recipients than are B10.D2 mice as skin grafts from them have a median survival time of 16.5 ± 1.1 days as against 13.5 ± 1.1 days for B10.D2 grafts.

A third donor strain, the black mutant B6.C-H(z1), abbreviated H(z1), was also used in grafting to B6AF_1 recipients. Cells from this mutant strain, originally described by Bailey et al. (2), are known to be capable of provoking proliferative lymphocyte responses in vitro in mixed cultures with cells from recipients of the genotype employed in our experiments. Skin grafts are rejected promptly. Nevertheless, no evidence of a serologically measurable immune response in this combination has so far been detected (3). These findings have been confirmed in our laboratory by using several techniques for measuring serological immunity by P. Sugarbaker and H. Winn, personal communication, so that it is clear that the response to the alloantigens involved in this strain combination, by comparison with most other strain combinations in which *H-2* incompatibility exists, is weighted heavily toward cellular immunity.

Operative Procedures

KIDNEY TRANSPLANTATION. Kidney transplantation was accomplished by direct vascular union as described previously (1). The recipients' kidneys were removed at the time of transplantation.

The survival of kidney transplants was evaluated by the survival of their recipients, by microscopic examination of sections prepared from them at the time of their removal on selected days (see below), and most sensitively by serial determination of blood urea nitrogen (BUN)[1] levels by using a standard urease micromethod. The latter determination was of particular value in the present experiments because it offered a means whereby transient effects could be detected and measured quantitatively. The normal range is considered to be from 15 to 30 mg/100 ml.

As described previously (1) about 30% of B6AF_1 recipients of B10.D2 kidneys die during the first 30 days. This occurs because of the effects of early rejection activity combined, in some instances, with the late consequences of technical imperfections such as some degree of ureteral obstruction. After about 1 mo the animals enter a relatively steady state and continuing attrition of recipients is low with only 20% more being lost by 100 days. A similar pattern of survival results when donor kidneys are obtained from the B10.BR and the H(z1) strains. Accordingly, the subjects for study in the present experiments were confined to recipients which had survived kidney transplantation for 4 wk or more and which had BUN levels in the normal range.

SKIN GRAFTING. Full thickness grafts of flank or ventral surface skin were placed on recipient beds on the lateral thoracic wall as described in detail by Billingham and Medawar (4).

LYMPHOID CELL SUSPENSIONS. "Sensitized" cells consisted of lymph node and spleen cells obtained from B6AF_1 animals which had rejected B10.D2 skin grafts and which had received an intraperitoneal injection of 10^8 B10.D2 lymph node and spleen cells from 4 to 7 days after graft rejection. The immunized cells were collected 7–14 days thereafter.

Spleen and lymph nodes were dissociated to form cell suspensions by gently passing them through a nylon sieve. The cells were collected in medium L-15 (Microbiological Associates, Walkersville, Md.), incubated with 0.14M NH_4Cl Tris buffer for 5 min at 37°C to lyse erythrocytes, and resuspended in the medium after two washings. The viability of cells in those suspensions employed was in excess of 80% as determined by trypan blue dye exclusion.

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[1] *Abbreviations used in this paper:* BCG, Bacille of Calmette and Guérin; BUN, blood urea nitrogen; C, complement; CMC, cell-mediated cytotoxicity; CY, cyclophosphamide.

HISTOLOGICAL PREPARATIONS. Tissue specimens were fixed in 10% neutral formalin in 50% ethanol, cleared in butanol, embedded in paraffin, and sectioned at 4 μm before staining with hematoxylin and eosin.

SEROLOGY. Complement-dependent cytotoxic antibody titers were determined in a two stage test as described previously (5). Trypan blue dye exclusion was employed to determine cell viability. A pooled antiserum against B10.D2 strain antigens was prepared from repeated bleedings of B6AF$_1$ female mice after at least four weekly injections of B10.D2 lymphoid cells. This pool had a cytotoxic titer of 1:512. Rabbit serum which had been exhaustively absorbed against mouse tissue was used as the source of complement (C) for infusion into mice.

^{51}Cr RELEASE ASSAY FOR CELL-MEDIATED CYTOTOXICITY (*CMC*). A modified version of the Brunner CMC assay, described previously (6), was used to detect the presence of killer cells in the spleens of B6AF$_1$ which had received B10.D2 kidney transplants. P815-X2 tumor cells were used as ^{51}Cr-labeled targets since they share H-2K^d antigenic determinants with the kidney donor. A mixture of lymphoid test cells and tumor target cells, at the appropriate ratios, was centrifuged and allowed to incubate for 16-18 h at 37°C. The cells were then resuspended and centrifuged and the amount of ^{51}Cr label released into the supernates was determined.

BCG AND CYCLOPHOSPHAMIDE. Bacille of Calmette and Guérin (BCG, Tice strain) was obtained from the University of Illinois Medical Center in vials containing 2-8 × 10^8 viable organisms. The BCG was reconstituted to 1 ml with sterile water, and an individual dose of 0.1 ml (approximately 2 × 10^7 organisms) was given intravenously. Cyclophosphamide (CY) from Mead Johnson Laboratories, Evansville, Ind. was dissolved in sterile water (20 mg/ml) and given i.p. in a dose of 4 mg/animal (160 mg/kg).

Results

Treatment with Sensitized, Recipient Strain Lymphoid Cells. Five B6AF$_1$ recipients of B10.D2 kidneys were used in this experiment. The transplants had been in place from 45 to 307 days and all were functioning normally. Two recipients received an intraperitoneal injection of 10^8 spleen and lymph node cells from B6AF$_1$ mice previously immunized to B10.D2 antigens. Two additional animals received a second injection of the same number of cells on the succeeding day, and a fifth animal received three daily injections of 1.5 × 10^8 cells from immunized donors. Fig. 1 shows the effects of these injections on the BUN levels of the recipients. At the first two dosage levels evidence of kidney damage was apparent by the 8th-15th day. The transplanted kidneys continued to support life in their recipients, however, and there was evidence of improvement in kidney function by about 3 wk after the injections. The transplant in the mouse which received the largest number of cells from immunized donors showed little response. At the doses of cells employed, it is apparent that definite damage to the transplant can result but that it did not progress and there was evidence of some late repair of function. With increasing doses of cells there was no marked increase in the degree of damage to the transplants.

Treatment with Alloantibody to Donor Antigens with and without Added Complement. Seven B6AF$_1$ recipients of B10.D2 kidneys were used in this experiment. Recipients received rabbit serum alone (0.5 ml) as a source of C, B6AF$_1$ anti-B10.D2 serum alone (0.5 ml), or the antiserum followed 6 h later by C. All injections were delivered intravenously. As inspection of Fig. 2 will reveal, neither antiserum nor C alone appeared to have any effect on kidney function under the conditions of these experiments except in one case (see middle panel) in which a sharp BUN elevation occurred by 5 h after C injection. This kidney went on to complete destruction. The recipient in this instance was known to have had an unusually high cytotoxic antibody titer to B10.D2 cells

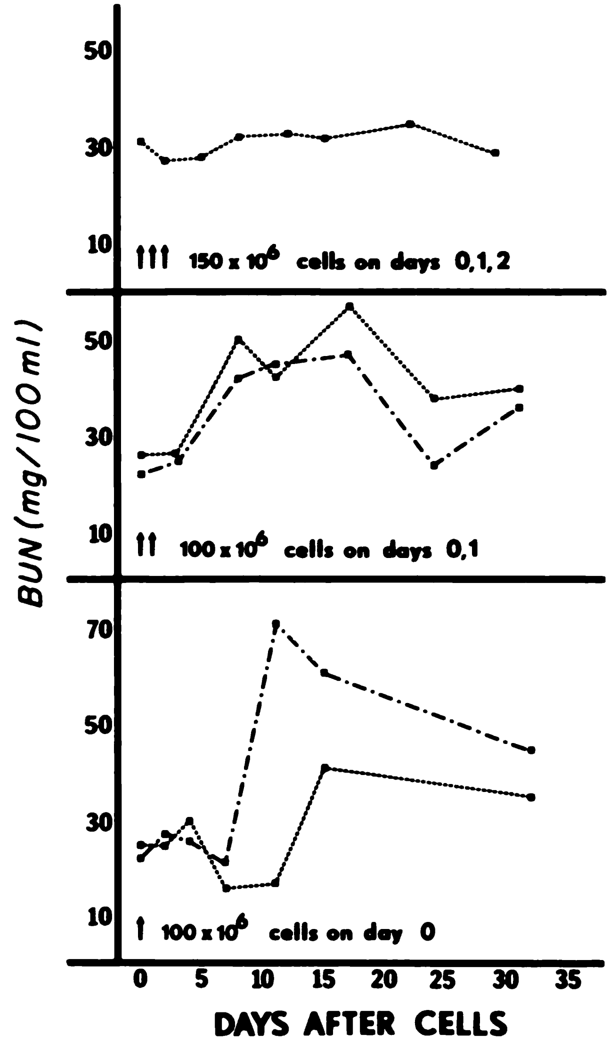

FIG. 1. The effect on transplant function of infusions of lymph node and spleen cells sensitized to donor (B10.D2) antigens. B6AF$_1$ recipients bearing normally functioning kidney transplants for 45–307 days received ascending doses, as indicated, of lymphoid cells intraperitoneally. Prompt evidence of kidney damage, as reflected in elevations of BUN levels, is apparent. Considerable improvement of renal function follows. With increasing cell dosage (within the limits tested) there is no evidence of increased kidney damage. Indeed, the least damage was seen at the highest cell dose.

(1:256). In five recipients it was found that antibody infusion, followed within a few hours by C, would cause very prompt kidney damage with striking elevation of BUN levels shortly after the C infusion. Thereafter kidney function returned to normal.

Treatment with Donor Strain Cells with or without BCG and CY. BCG and CY were employed according to a protocol originally described by Mackaness

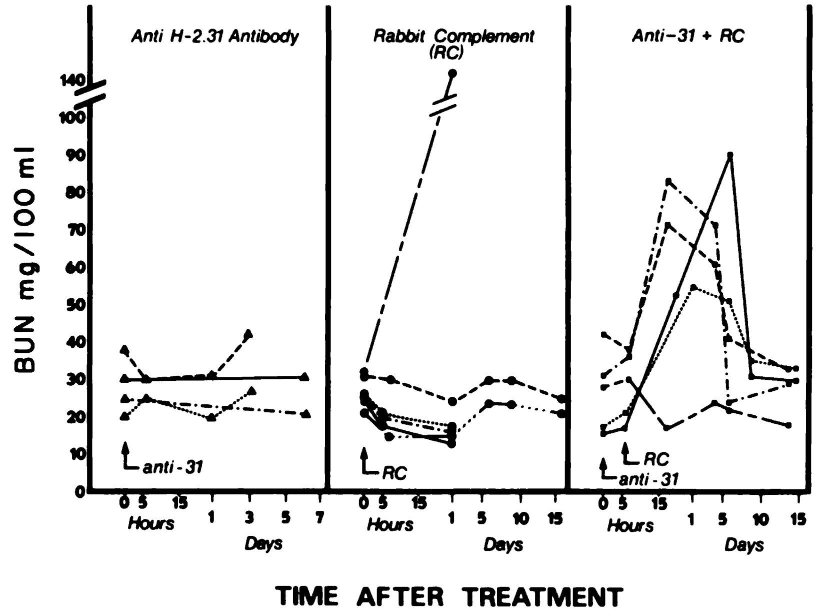

FIG. 2. The effect of intravenous infusions of antibody and C on the function of long-surviving B10.D2 kidneys transplanted to B6AF$_1$ recipients. In the left panel the BUN records of four transplant recipients are shown. At time 0 all received 0.5 ml of an antibody directed to donor antigens (see text). This did not influence the function of the transplanted kidneys. In the middle panel six recipients received injections of C (0.5 ml of rabbit serum absorbed with mouse cells). No effect was seen except in the case of one recipient known to have an unusually high titer of antibody to donor antigens. In the right panel, antibody infusion to five recipients was followed in 6 h by C. Impairment of kidney function was apparent by elevation of BUN levels in four of the five animals within 24 h. This damage was transient and kidney function after this treatment returned to normal in all animals.

and Lagrange which they devised to alter the state of specific unresponsiveness to sheep erythrocytes which can be induced in mice by large doses of this antigen (7).

CONTROL. TREATMENT WITH RECIPIENT CELLS ALONE. This control was performed to rule out a possible nonspecific effect resulting from the injection of large numbers of spleen and lymph node cells which might lodge in the transplanted kidney or cause damage to the kidney in some other nonspecific fashion. Accordingly, three B6AF$_1$ mice, which had received B10.D2 kidney transplants at least 4 wk previously, were injected with 1.5 × 10^8 normal B6AF$_1$ lymph node and spleen cells intraperitoneally on 2 successive days. No effect on BUN levels was observed during a subsequent month of observation.

TREATMENT WITH DONOR STRAIN CELLS ALONE. Four B6AF$_1$ kidney recipients received 1.5 × 10^8 pooled B10.D2 lymph node and spleen cells intraperitoneally on 2 successive days. An incresae in BUN levels was observed in all mice by the 6th day after the initial cell injection. The damage induced in the kidney transplants was transient, however, as BUN values steadily returned to near normal levels within 4 wk (see Fig. 3). Two of these four recipients underwent

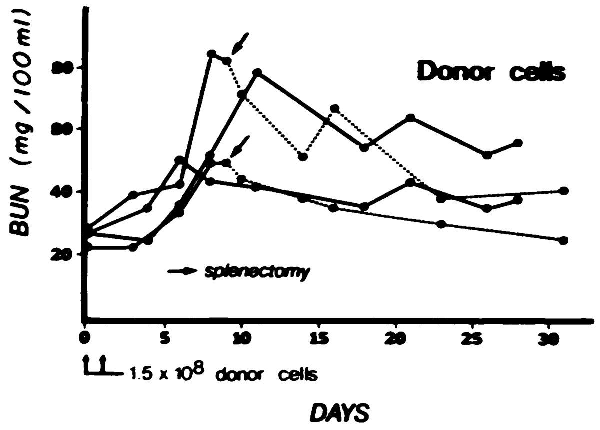

FIG. 3. The effect of donor cells on transplant function. Intraperitoneal injections of 1.5 × 10^8 donor strain lymph node and spleen cells on 2 succeeding days resulted in elevations of BUN levels in all four B6AF$_1$ mice which were long-term recipients of B10.D2 kidney transplants. The damage provoked by these cells tended to be repaired gradually. Injection of the same number of recipient strain cells to three other animals (not shown) failed to induce any evidence of transplant damage.

splenectomy at the height of the induced response so that their spleens could be assessed for the presence of killer cells as measured by ^{51}Cr release from P815-X2 target cells (see Fig. 4). Evidence of only a sluggish immune response was revealed by this test especially as compared to the level of cell-mediated cytotoxicity found in the spleens of animals which had recently received and rejected skin grafts. It was also of some interest that removal of the spleen at this point did not alter the course of the response as compared to the other animals tested.

TREATMENT WITH BCG AND CY. BCG (2 × 10^7 organisms) was given intravenously to two B6AF$_1$ mice with normally functioning B10.D2 kidney transplants at 8–15 wk. The BUN levels remained stable thereafter and were not influenced adversely when an injection of CY (4 mg) was given 20 days later (see Fig. 5).

TREATMENT WITH CY AND DONOR CELLS. Five B6AF$_1$ recipients of B10.D2 kidneys received injections of 4 mg of CY followed 2 days later with the first of two daily intraperitoneal injections of 1.5 × 10^8 B10.D2 spleen and lymph node cells. By the 10th–12th days after the initial cell injection four out of five of the treated mice showed marked BUN elevations and three of these animals were sacrificed, two with BUN levels of over 200 mg/100 ml. The fourth animal recovered considerably from the damage incurred and survived for several months. The remaining transplant showed only a minor degree of damage during the observation period (see Fig. 6).

TREATMENT WITH BCG AND DONOR CELLS. Four B6AF$_1$ recipients of B10.D2 kidney transplants received injections of 2 × 10^7 BCG organisms from 42 to 144 days after transplantation. On the 22nd and 23rd days after BCG treatment, they received intraperitoneal injections of 1.5 × 10^8 B10.D2 lymphoid cells. All of these animals showed a sharp rise in BUN by the 7th–8th day after the initial cell

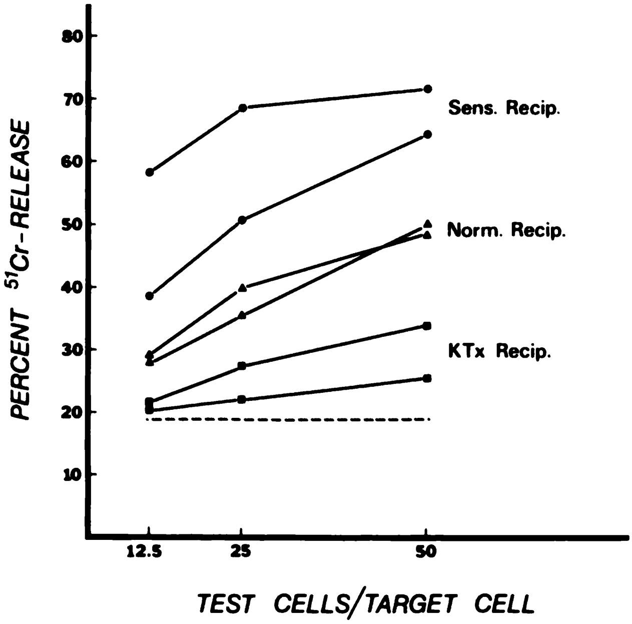

FIG. 4. Normal B6AF$_1$ animals, B6AF$_1$ animals which had recently rejected B10.D2 skin grafts, and B6AF$_1$ recipients of B10.D2 kidneys were injected i.p. with 1.5 × 10^8 B10.D2 lymphoid cells 8 and 9 days before removal of their spleens. Increasing numbers of spleen cells from each animal were mixed with ^{51}Cr-labeled P815-X2 cells and incubated for 16 h before the supernates were harvested. Spontaneous release (- - -) = 19.8%. The capacity of kidney transplant recipients to produce cytotoxic cells in vivo under these conditions was less than that of normal mice and markedly less than that specifically immunized animals.

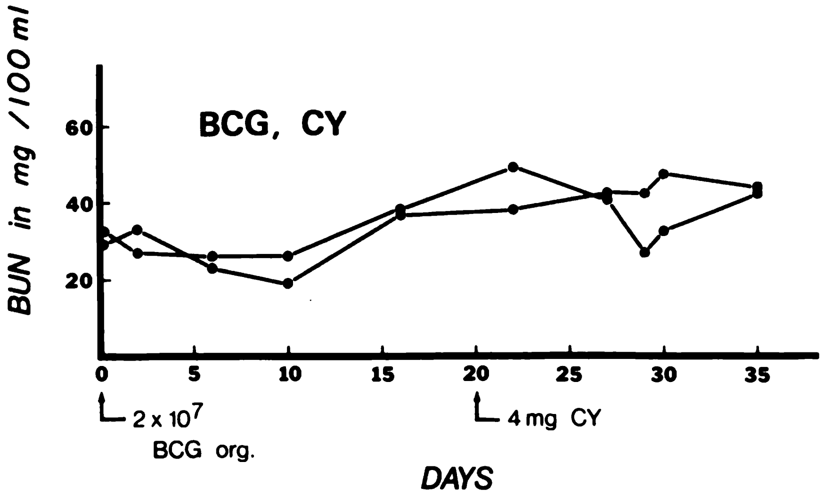

FIG. 5. The administration of BCG and CY in the dosages indicated failed to produce any evidence of renal damage in two control B6AF$_1$ mice which were long-term recipients of B10.D2 kidney transplants.

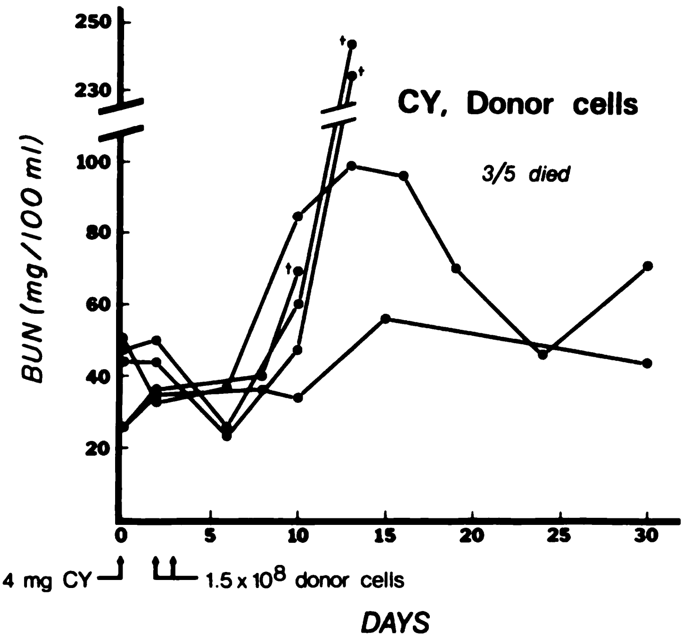

FIG. 6. Treatment of five B6AF$_1$ recipients of B10.D2 kidney transplants with CY followed by two daily intraperitoneal injections of donor strain lymphoid cells rapidly provoked marked evidence of renal damage in all but one animal the BUN of which showed only moderate elevation. Three of the five animals became moribund and were sacrificed, whereas two recovered.

injection. Three went on to severe uremia and were sacrificed when moribund. The fourth animal survived with a BUN at a slightly elevated level (see Fig. 7).

TREATMENT WITH BCG, CY, AND DONOR CELLS. As further controls for this particular experiment, two unilaterally nephrectomized B6AF$_1$ mice received injections of BCG and of CY 20 days later in the standard doses described. 2 and 3 days after this they received intraperitoneal injections of 1.5 × 10^8 B10.D2 lymphoid cells. Their BUN levels remained normal throughout this period and for at least 30 days thereafter.

Six B6AF$_1$ recipients of B10.D2 kidney transplants received the "triple treatment" of BCG, CY, and donor strain lymphoid cells according to the schedule described above. By the 6th day after the first injection of B10.D2 cells, the BUN levels of all animals had begun to rise sharply. All transplants went on to complete cessation of function and their recipients were sacrificed as they became moribund (see Fig. 8).

At sacrifice the spleens of three of these mice were removed and were tested in vitro for cell-mediated cytotoxicity to donor antigens by evaluating their ability to cause release of label from ^{51}Cr-tagged P815-X2 mastocytoma cells. As can be seen in Fig. 9, active killing of target cells was demonstrable in all cases even at low lymphocyte to target cell ratios. Such killing is similar to that produced by cells from mice which have recently rejected skin grafts, but has never been observed with cells from long-term kidney transplant survivors (1).

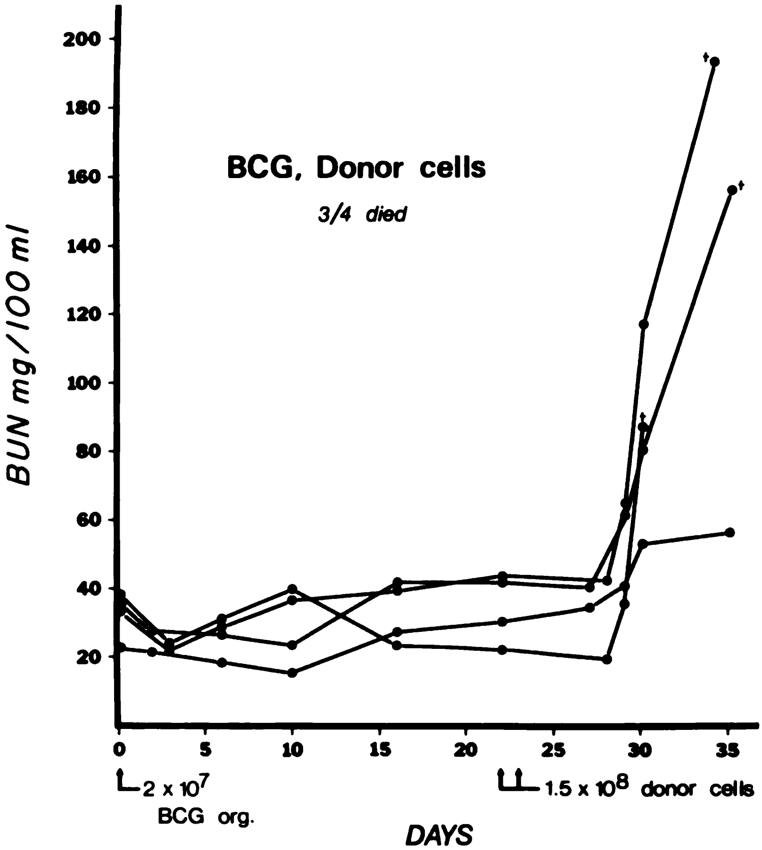

FIG. 7. Treatment of four B6AF$_1$ recipients of B10.D2 kidneys with BCG followed 22 and 23 days later with two intraperitoneal injections of 1.5 × 10^8 donor strain lymphoid cells. Evidence of transplant damage was apparent by 7 days in all animals. In one of these, the damage failed to go to completion and the animal survived.

No cytotoxic antibody was detected in serum from any of these three animals at the time they were sacrificed.

To determine whether or not the allogeneic cell inoculum must share foreign specificities with the kidney transplant to induce a damaging effect against it, two B6AF$_1$ mice were employed which had born normally functioning B10.D2 kidney transplants for 69 days or more. Each was treated with BCG and CY as above, but the cell infusions were derived from A.SW donors. Neither animal showed evidence of kidney damage or alteration in its state of apparent well being during a period of several weeks of observation after the completion of the treatment.

The "triple treatment" regimen was also applied to four B6AF$_1$ recipients of B10.BR kidney transplants. Once again, by the 7th day after the first donor strain cell injection, the BUN levels of all animals became distinctly elevated from previously normal levels and all animals became fatally uremic by the 9th day (see Fig. 10). A similar result was obtained with three B6AF$_1$ recipients of

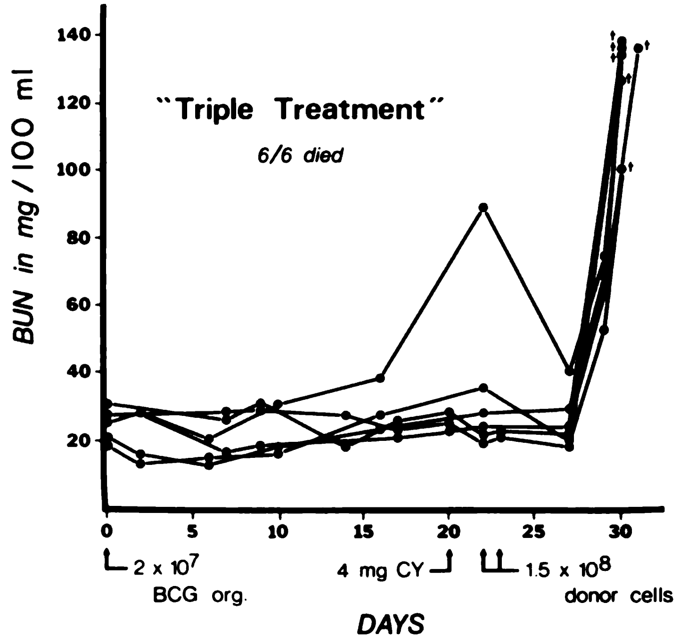

FIG. 8. Six B6AF₁ recipients of B10.D2 kidney transplants received BCG, CY, and donor cells in the "triple treatment" program as indicated. All manifested severe renal damage by the 7th or 8th day and either died or required sacrifice in a moribund state.

H(zl) kidney transplants. All mice became markedly uremic by the 6th day and all were sacrificed in a preterminal state by the 12th day after the receipt of donor strain cells.

Sections of fixed renal tissue were examined microscopically in a number of instances and the morphologic findings are summarized in Table I. Transplants from untreated recipients showed minimal focal mononuclear and plasma cell infiltrates that were localized principally around the vessels in about 5% of the cortex. Large arteries tended to show intimal thickening and mononuclear infiltration, and the glomeruli manifested moderately extensive focal and segmental scarring. No mononuclear cell accumulation was found on the endothelial surfaces. Kidney transplants from B10.BR and B10.D2 donors were examined microscopically 6-7 days after donor cells were given in the "triple treatment" regimen. In sharp contrast to the appearance of kidneys from untreated recipients, all of these showed an intense, diffuse infiltrate of mononuclear cells in the interstitium and focally within tubules (Fig. 11). The most striking change was the accumulation ("sticking") of mononuclear cells on arterial endothelial surfaces throughout the arterial system. These cells resembled lymphoblasts with basophilic cytoplasm and occasional nucleoli. Cells with indented nuclei, consistent in appearance with monocytes, were also present as well as occasional neutrophils. Many arterial lumena contained a loose meshwork of pale eosinophilic fibrillar material. The endothelial cells were occasionally basophilic; sometimes no endothelial nuclei were seen in an entire vessel cross section, indicating endothelial loss. However, necrotic endothelial cells or

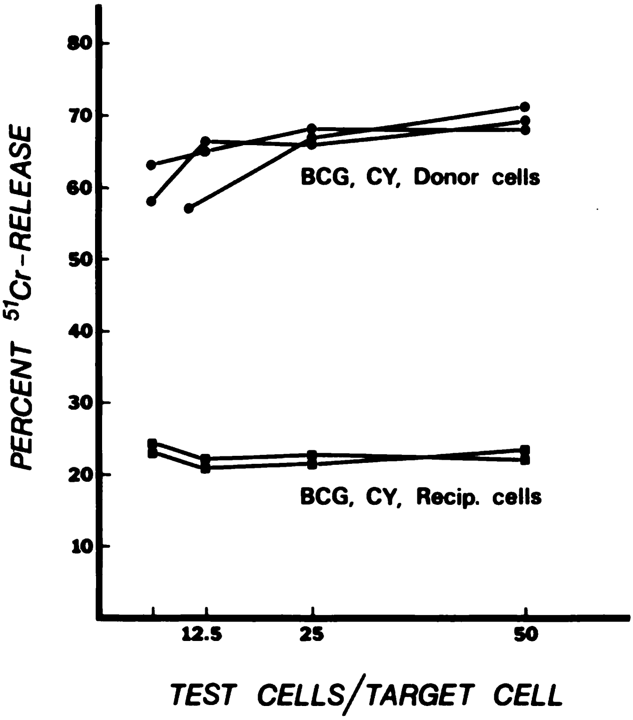

FIG. 9. B6AF$_1$ recipients of B10.D2 kidneys were treated with BCG, CY, and donor or recipient strain cells as described in the text. Spleens from these animals were removed 8 days after the second cell injection and the splenic cells were mixed in increasing numbers with ^{51}Cr-labeled P815-X2 cells. The supernates were harvested after a 16-h incubation period. Spontaneous release = 19.5%. Treatment with donor strain cells resulted in the induction of high levels of cell-mediated cytotoxicity which were not seen with recipient strain cells.

frank thrombosis were not identified. Glomeruli showed segmental damage with loss of architecture which was not significantly more severe than that seen in untreated animals. Allografts from animals which had received BCG and cells alone resembled in all respects those that received CY in addition.

Discussion

These experiments shed some additional light on the nature of the equilibrium which is arrived at spontaneously between graft and host in the strain combinations studied. The fact that donor antigens remain on display on the surfaces of donor cells is affirmed by their vulnerability to the effects of passively conferred immunity, either humoral or cell mediated. Although we have no information regarding possible changes in the density of such cell surface antigens, they appear to persist widely throughout the parenchyma of the kidney. In particular, the intense and acute reaction which occurs against the endothelium of vessels in transplants after treatment of their recipients with BCG, CY, and donor strain lymphoid cells is evidence against the notion that graft endothelium is replaced by recipient cells with a period of 1 or 2 mo.

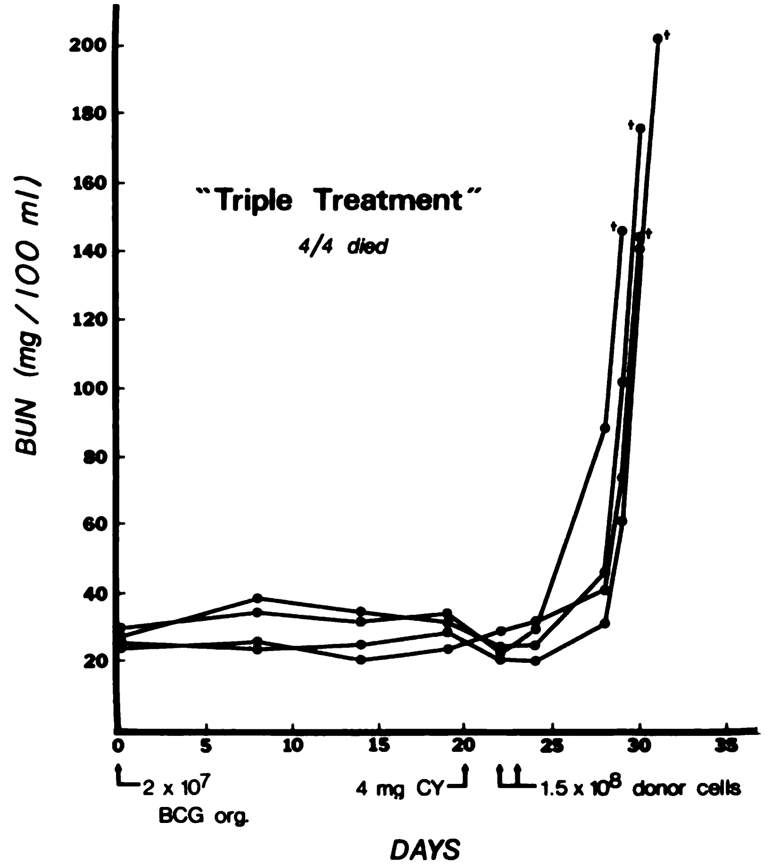

FIG. 10. Long-term B6AF$_1$ recipients of B10.BR kidneys in which relatively little histoincompatibility is involved underwent the "triple treatment" regimen outlined in the text. Without fail all showed prompt evidence of rejection which rapidly destroyed all the transplants. Primary kidney transplants in this strain combination are rarely rejected (see text and reference 15).

TABLE I

*Morphologic Features of Rejection in Kidney Transplant Recipients after Receiving BCG, CY, and Donor Cells**

Kidney and cell donor strain	Treatment	Number of animals	Allograft age	Days after donor cells	Infiltrate		Glomerular damage
					Interstitial	Endothelial	
			days				
B10.D2	None	12	39-181	–	0/+	0	+/+++
B10.D2	BCG/CY/Cells	3	132-347	7	+++	+++	++
B10.D2	BCG/Cells	2	76-182	11-12	+++	+++	+++
B10.BR	BCG/CY/Cells	3	61-72	6-7	+++	+++	+++

The findings are described in detail in the text.

* The extent and intensity of the changes were scored qualitatively from 0 to ++++.

The fact that no apparent damage to transplants was inflicted by the infusion of antibody specifically directed against graft antigens without the further addition of rabbit C probably attests to the relative inefficiency of mouse complement in such reactions. A similar result has been reported by Koene et al., who found that skin allotransplants between mice of the same strains as those used in many of our experiments (B10.D2 to $B6AF_1$) will be destroyed acutely only if infusions of antibody to the donor strain are supplemented with rabbit complement (8). Another similar result was reported by French who found that acute destruction of rat kidney transplants, surviving as a consequence of infused antibody directed toward donor antigens (immunological enhancement), could be induced by the administration of an effective complement in the form of guinea pig serum (9). It is not clear exactly why the effects which are noted on the function of our transplanted mouse kidneys after antibody and C treatment do not generally result in irreversible changes. The most likely explanation can probably be found, however, in the availability of sufficient quantities of all of the components required for the full expression of an inflammatory reaction rather than any lack of susceptibility of transplanted kidneys to such injury. Previous studies performed in our Unit of the damaging effects of infused antibody on surviving skin grafts established that although the ready availability of polymorphonuclear leukocytes and complement are important to the onward progress of antibody mediated destruction, other factors can participate in the process (10), W. Soper and H. J. Winn, personal communication.

The donation to transplant recipients of large numbers of lymphoid cells from mice highly immunized to the kidney transplant antigens was demonstrated to produce a definite impairment in renal function but only transiently. It was somewhat surprising that no definite increase in the destructive potential of cells transferred from preimmunized donors was seen with increasing doses. This result may be similar to those reported by Batchelor and Welsh and Bowen et al. (11, 12). They found that rats, which can be made to accept transplanted kidneys for long periods by early treatment with antibody against donor strain antigens, will enter a "steady state" during which no further treatment is required for the continuing survival of the transplants. The transfer of large numbers of specifically immunized lymphoid cells to these survivors, or even the parabiosis of such recipients with syngeneic animals immunized to the kidney transplant antigens, failed to bring about rejection of the transplanted tissue. In our system the available evidence suggests that an important point at which interference with the normal evolution of the immune response to transplanted kidneys occurs is in the generation of killer T cells (1). It may be that even in cell populations from preimmunized donors, the number of active killer cells is relatively small and that the recruitment of additional killer cells is inhibited under the conditions prevailing in long-term transplant recipients.

The above considerations make it particularly striking that the cell-mediated immune processes of the host can be called into such vigorous action by treatment with donor antigens reinforced by BCG, CY, or both. These treatments can result in the complete rejection of a transplant which the host is incapable of rejecting either through its own active processes or even after

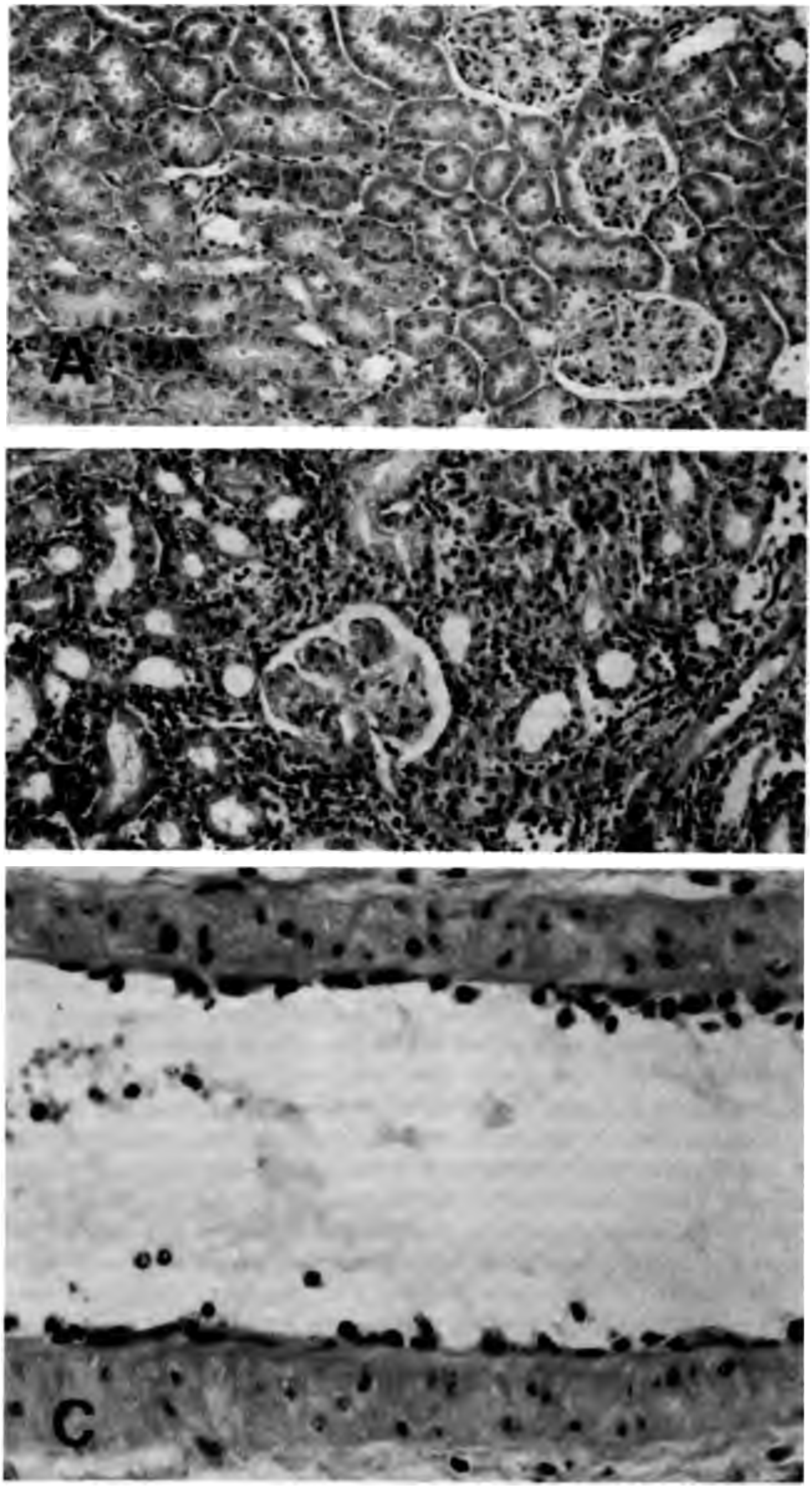

FIG. 11

receiving large numbers of cells from specifically immunized donors. Evidence that these regimens result in the generation of a vigorous cellular response lies both in the rapid appearance of killer cells in recipient spleens and lymph nodes and in the rich cellular infiltrate which is found in the transplanted kidneys as they cease to function. Under the conditions tested so far, an indispensable part of the treatment regimen is the inclusion of a disseminated wave of donor specific antigens. Although this stimulus, on its own, will result in a readily detectable immune response to the transplant, the response does not cause the complete rejection of any transplants and loses its force in a few days. As reported previously (13), the application of a donor strain skin graft to a surviving kidney transplant recipient results in no discernible reaction against the transplanted kidney. Indeed, donor strain skin grafts in this system survive for prolonged periods and their rejection, when it occurs, is very gradual. Nevertheless, the vigor of the acute rejection which can be called forth especially by the "triple treatment" regimen, in which all surviving transplants are completely destroyed in 7-8 days, is similar in tempo to an accelerated reaction which would ordinarily occur only in the presence of pre-existing immunity. Thus, even though kidney transplant recipients have been shown to be quite deficient in "memory cells" by in vitro tests, by the time they have traversed the three and one half weeks required for the "triple treatment" program they behave as though preimmunized. Even though the administration of donor cells appears to be essential to the increase in the immune response which we have observed, supplementary treatment with CY and BCG has been required to provoke rejection in all transplants. It is impressive that this treatment is also effective even in the presence of the weaker immunogenetic disparity represented by B10.BR transplants to $B6AF_1$ recipients.

The mechanisms to be considered as possible contributors to this sharp change in the balance between transplant and host could lie in several areas, and each agent in the treatment program could play one or more roles. Among the factors to be considered are: (*a*) influences on suppressor cells, (*b*) the restoration of responsive cells to a population made deficient in them through clonal deletion or inactivation, or (*c*) effects on serum factors inhibitory to the evolution of a fully effective state of cell mediated immunity. Our experimental observations are entirely consistent with those of Mackaness and Lagrange (8) who designed the BCG and CY treatment regimen we have used. To restore delayed hypersensitivity reactions to their animals additional treatment with antigen was required just as we find in our system. The precise requirements for the antigenic stimulus and timing of delivery for the components of the

FIG. 11. Demonstration of morphological changes in transplanted kidneys after "triple treatment". (A) An allograft from an untreated mouse 134 days after transplantation. Little cellular infiltrate is present (no. 535, × 190). (B) An allograft 132 days after transplantation. The mouse received BCG, cyclophosphamide, and donor cells. The last dose of cells was given 7 days before sacrifice. The kidney shows an intense, widespread interstitial monuclear infiltrate and tubular damage. The segmental obliteration of glomerular architecture illustrated was also found in the controls (no. 571, × 190). (C) Longitudinal cross section of an artery in the allograft in Fig. 11B. Numerous cells, largely mononuclear, have accumulated along the endothelial surface. Focal loss of endothelium has occurred (no. 571, × 430). All sections are from B10.D2 to $B6AF_1$ transplants.

treatment have not been established in kidney transplant recipients. Mackaness and Lagrange postulated that the influence of CY in their system was attributable to its selective inactivation of B cells which thus resulted in a reduction in antibody levels and consequently in antibody feedback inhibition of T-cell activity. BCG was believed to interfere with "serum blocking factors".

Only a preliminary consideration of the various mechanisms is possible from the experiments reported in this communication and the accompanying paper (1). We have demonstrated that our animals do not manifest marked T-cell unresponsiveness to donor antigens, i.e., they are not tolerant in the conventional sense, and no direct evidence to support the presence of suppressor cells has been uncovered. Thus, however plausible it might be, we cannot attribute the action of CY to a depletion of suppressor T cells as it is reportedly capable of producing in some systems (14). Humoral antibody with complement-dependent cytotoxic activity for donor cells can be detected in low titers, especially in the early weeks after transplantation, in some B6AF$_1$ recipients of B10.D2 kidneys, but current evidence makes it unlikely that suppression of T-cell function by antibody directed to H-2 determined histocompatibility antigens could be a full explanation for the long-term survival of these transplants. This evidence includes the fact that kidneys transplanted from H(zl) donors to B6AF$_1$ recipients survive very well even though no humoral antibody to donor cells in this strain combination can be produced by a variety of methods. Nevertheless, there is evidence for the presence of a substance in the sera of long-term recipients which inhibits the ability of nonimmune cells to differentiate into effector cells (1). We have not yet made efforts to determine whether this substance is eliminated by the "triple treatment" regimen, but it would seem logical to expect that this might be at least one of its results.

Whatever the mechanisms may be for the generation of this marked immunological reaction, they do not appear to result in potentiation of the humoral arm of the response since no evidence of cytotoxic antibody production could be detected after the "triple treatment". These results serve to emphasize the complexity of the immune relationship which can exist between a vascularized mass of cells bearing foreign surface antigens and its host. That this immune balance can be so decisively upset should stand as a point of caution in clinical organ transplantation but perhaps as a source of encouragement to oncologists.

Summary

Various modes for producing the specific immune destruction of surviving kidney transplants between mice were tested in these experiments. Kidney transplants among mice which were incompatible at the *H-2* locus but which were surviving in excellent condition for several weeks without immunosuppression were utilized as subjects for these experiments. When immune damage to these surviving organs resulted from the treatments being tested it was readily detectable by changes in the blood urea nitrogen levels of their recipients. The treatments included means of heightening immune reactivity to donor antigens passively by the transfer of either specifically activated cells, immune serum, or by the active generation of increased responsiveness.

Infusions of an antiserum specifically reactive with the histocompatibility

antigens of the transplant were ineffective in causing damage to the kidney unless exogenous complement, in the form of rabbit serum absorbed with mouse tissue, was also given. The injection of lymph node and spleen cells from recipient strain mice which had been highly immunized to donor antigens caused definite but transient damage to transplanted kidneys.

Stimulation of recipient responsiveness by additional donor tissue antigens presented as skin grafts was ineffective although intravenous injections of lymphoid cells provoked an evanescent reaction. Combining donor strain lymphoid cell treatment with a prior injection of cyclophosphamide (4 mg, 2 days before cell injection) and/or treatment with Bacille of Calmette and Guérin (2×10^7 organisms, 22 days before cell injection) caused an intense and specific immune response to donor antigens with rapid onset of transplant damage. The maximal effect observed followed the combined use of all three agents in which case every transplant was fully rejected by 7 or 8 days after donor cell injection.

These results demonstrate that an otherwise stable balance between an incompatible transplanted organ and its host can be decisively upset by treatments which provoke a heightened, specific immune response. The conferral of immunity passively by transferring either serum or lymphoid cells from sensitized donors was much less successful in causing damage to transplants. This information must be interpreted in the light of the evidence we have presented in an accompanying paper (1) that the maturation of fully competent killer T cells is retarded in long-term kidney transplant recipients and that no direct evidence of suppressor cell activity can be found in them. The fact that only transient damage to transplants occurred even after the transfer of large numbers of immunized lymphoid cells, however, has made us refrain from dismissing altogether the participation of an active suppressor mechanism.

We would like to acknowledge the effective aid of Ms. Susan W. Ayres in the preparation of the manuscript and the helpful comments of Dr. Henry J. Winn.

Received for publication 29 December 1977.

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GENETIC STUDIES IN NZB MICE

I. Spontaneous Autoantibody Production

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NZB mice spontaneously produce a variety of autoantibodies and serve as a model of autoimmunity (1). As they age, NZB mice produce antibodies to erythrocytes, thymocytes and T cells, and nucleic acids, especially single-stranded DNA (ssDNA)[1] (2).

Naturally occurring thymocytotoxic autoantibodies (NTA) have been found early in life in NZB mice (3). These antibodies are present in almost all NZB mice by 3 mo of age, and are associated with an age-dependent loss of T cells in these mice (4, 5). Sera with high titer NTA activity were shown to affect a subpopulation of T cells by selectively decreasing the response of normal spleen cells to the mitogen Concanavalin A (6). NTA appear to be functionally important. These antibodies impair cellular immune responses (7) and cause a loss of suppressor T cells (8). Although other mouse strains may produce NTA later in life, the prevalence and titer of NTA are much lower than in NZB mice (3).

Because both NTA and antinucleic acid antibodies are characteristic of NZB mice and may be important in the pathogenesis of autoimmunity (9), we set out to investigate the mode of inheritance of the propensity for development of these autoantibodies.

Reciprocal F_1 hybrids between the DBA/2 and NZB strains were studied to determine whether or not the inheritance was dominant or regulated by a gene on the X chromosome. Further studies were performed in backcrosses. The DBA/2 strain was chosen as one of the parental strains because: (*a*) it is H-2^d like NZB mice (10), (*b*) like NZB mice it expresses GP70 viral protein antigens in large quantities on the surface of mononuclear cells (T. M. Chused and H. C. Morse, III, personal communication) and (*c*) it does not spontaneously produce large quantities of either NTA or anti-ssDNA (11, 12). Thus, the possibility of an *H-2*-linked gene controlling either NTA or anti-ssDNA production could be eliminated, and the contribution of GP70 expression minimized. We found that the hybrids were intermediate between high producing (NZB) and low producing (DBA/2) parents with regard to both NTA and anti-ssDNA production. In preliminary studies (13), NTA were produced by female but not male F_1 hybrids. Castration of the males abolished this sex difference; castrated males had the same incidence and titer of NTA as did their female littermates. This was consistent with the notion that male sex hormones can exert a negative immunoregulatory effect (14). We therefore concluded that further genetic studies of the regulation of autoantibody production must deal directly with the problem of sex effects. It appeared to us that the best way to perform a genetic analysis of a sex-influenced trait was to test gonadectomized progeny.

In this paper we report a study of the mode of inheritance of the propensity for production of thymocytotoxic autoantibodies. Because sex hormones influenced the expression of these antibodies, a genetic study was carried out utilizing go-

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[1] *Abbreviations used in this paper:* C, complement; NTA, naturally occurring thymocytotoxic autoantibodies; ssDNA, single-stranded DNA.

nadectomized offspring to better determine the precise genetic mechanisms in the absence of the modulating influence of sex hormones. In addition, we studied the genetic control of antibodies to ssDNA in the same mice. We found that inheritance of the tendency to develop NTA is not linked to the tendency to develop ssDNA antibodies. However, each is primarily controlled by a single gene locus.

Materials and Methods

Mice. – All mice were derived from colonies at the National Institutes of Health Animal Production Unit. NZB/N and DBA/2N mice were reciprocally crossed in our laboratories to produce (NZB X DBA/2)F_1 and (DBA/2 X NZB)F_1 progeny. Male and female F_1 hybrids were backcrossed to both parental stains. Virgin mice of both sexes from parental strains, F_1 hybrids, and backcrosses were chosen for surgery at 4–5 wk of age. Animals were injected intraperitoneally with sodium pentobarbital before surgery. In female mice, the ovaries were removed through two bilateral flank incisions (15). In males, both testes were removed through a single scrotal incision. The vas deferens was tied, and each testis and epididymis was removed. Vasectomy of select males were performed by vasoligation above and below the transection.

Sera. – Mice were bled at 1 yr by orbital sinus puncture under light ether anesthesia. The blood was allowed to clot at room temperature for about 2 h, after which the sera were removed, centrifuged to remove cellular debris, and stored at −20°C until used. Because of a laboratory accident (freezer breakdown) sera from gonadectomized (NZB X DBA/2)F_1 females were not available for study with regard to ssDNA antibodies and serum IgM concentrations.

NTA Assay. – The presence of NTA was measured as described previously (16), with only minor modifications. Thymocytes from 4- to 6-wk-old C57BL/6N mice were incubated with 30 μCi of ^{51}Cr per 10^7 cells for 30 min at 37°C in RPMI-1640 medium. Labeled thymocytes (5×10^4) were added to 50 μl of serially diluted test serum and incubated for 30 min at room temperature followed by 30 min at 4°C. Cells were centrifuged, washed twice in medium, and incubated with 50 μl of a 1:5 dilution of rabbit complement (previously absorbed with mouse thymocytes) for 30 min at 37°C. The suspension was then centrifuged, and 50 μl of supernate removed and counted in a gamma spectrometer. The positive control was a pool of NZB sera with known NTA activity; the negative control consisted of sera from 1-yr-old female DBA/2 mice. Maximum ^{51}Cr release was obtained by freeze-thawing three times. Cytotoxicity of complement (C) alone was also determined. Percentage cytotoxicity was calculated as

$$\frac{(\text{cpm test serum} - \text{cpm C})}{(\text{cpm freeze-thaw} - \text{cpm C})} \times 100.$$

All sera were assayed in duplicate with good reproducibility. The cytotoxic titer was the last dilution giving >50% ^{51}Cr release. A positive titer in this assay is 1:4.

Measurement of IgM Levels. – Serum IgM concentrations were measured by radial immunodiffusion in Meloy immunodiffusion plates (Meloy Laboratories, Inc., Springfield, Va.). Sera were added to wells cut in agarose gel impregnated with monospecific IgM antibody. The plates were incubated for 18 h at room temperature in a humidified atmosphere, and the diameter of the precipitin ring measured and compared to known standards run on the same plate.

Measurement of Anti-ssDNA Antibodies. – A previously described modified Farr technique was employed (17). The reaction mixture (100 μl) contained 50 ng ^{14}C-labeled heat-denatured *Escherichia coli* DNA (50,000 dpm/μg) and 25 μl of test serum (previously heated to 56°C for 30 min) in borate buffer, pH 8.0. The mixture was incubated for 1 hr at 37°C, then refrigerated overnight at 4°C, after which an equal volume (100 μl) of 70% saturated ammonium sulfate was added. The mixture was then incubated at 0°C for 1 h and centrifuged at 1,000 *g* for 20 min. 100 μl of supernate was removed, and the amount of radioactivity determined in a liquid scintillation counter. Positive and negative controls were run in duplicate or triplicate with test sera. A single batch of fetal calf serum (FCS) was always assayed as a negative control for nonspecific binding of the ligand. The percent binding by the FCS was subtracted from each test serum. A test was considered positive if the binding was greater than the highest binding ever recorded for FCS as well as 2 SD greater than the mean of replicate runs of normal mouse sera.

TABLE I
Study of NTA Production in Intact NZB, DBA/2, F_1, and Backcross Mice

Intact animal	Sex	Positive NTA test		Combined sex frequency
		No./total	Frequency	
NZB	F	14/16	0.88	0.91
NZB	M	16/17	0.94	
DBA/2	F	1/14	0.07	0.04
DBA/2	M	0/14	0.00	
(NZB × DBA) F_1	F	13/28	0.46	0.24
(NZB × DBA) F_1	M	0/26	0.00	
F_1 × NZB	F	13/24	0.54	0.48
F_1 × NZB	M	9/22	0.41	
F_1 × DBA/2	F	1/30	0.03	0.09
F_1 × DBA/2	M	4/25	0.16	

Results

Prevalence of NTA. – Table I shows the prevalence of NTA in the two parental strains, the F_1 hybrids, and the backcrosses to both parental strains. In the right column of Table I, the incidence of NTA is reported without regard to sex. The F_1 hybrids have a higher incidence of NTA than does the DBA/2 parental strain. Backcrosses to the NZB strain have an intermediate incidence of NTA between the NZB parental strain and the F_1 hybrids, whereas the backcrosses to the DBA/2 strain have as low an incidence as the DBA/2 parental strain. The data on the right side of Table I do not suggest a simple genetic mechanism. In the center of Table I the incidence of NTA is reported by sex. In the case of the F_1 hybrids, the males had a significantly lower incidence of NTA than did the females ($P < 0.001$). NTA production is not an X-linked trait because F_1 males of both reciprocal crosses did not produce NTA (13, and unpublished data). The sex difference in the incidence of NTA is attributed to sex hormones, and can be abolished by castration (13). No meaningful genetic analysis could be made from these findings of intact animals. Therefore, the genetic control of NTA production in castrated offspring was studied.

Effect of Gonadectomy on the Prevalence of NTA. – In Table II the incidence of NTA in gonadectomized male and female mice is shown. Castration of the males increased the incidence of NTA and thereby removed the sex differences observed in intact F_1 mice. The differences between males and females with regard to NTA incidence in gonadectomized animals are insignificant; therefore, the sexes were combined. In the right-hand column and at the bottom of Table II the expected incidence of NTA is shown; the calculations are based on the observed NTA incidence in gonadectomized parental strains. This calculation assumes that: (*a*) each parental strain has a distribution of NTA production (incomplete penetrance), (*b*) each parental contribution to offspring NTA production is equivalent to a random sample of the parental distribution, and

TABLE II

Study of the Genetic Factors Influencing NTA Production by Analysis of Gonadectomized Offspring

Gonadectomized animal	Sex	Positive NTA Test		Combined sex frequency	Expected frequency*	χ^2 (*P*)
		No./total	Frequency			
NZB	F	9/10	0.90	0.94	–	
NZB	M	8/8	1.00			
DBA/2	F	1/9	0.11	0.09	–	
DBA/2	M	1/13	0.08			
(NZB × DBA) F_1	F	9/15	0.60	0.52	0.52‡	0.00 ($P > 0.75$)
(NZB × DBA) F_1	M	13/27	0.48			
F_1 × NZB	F	8/12	0.67	0.71	0.73§	0.03 ($P > 0.50$)
F_1 × NZB	M	7/9	0.78			
F_1 × DBA	F	4/14	0.29	0.21	0.31‖	1.44 ($P > 0.50$)
F_1 × DBA	M	2/15	0.13			

* Expected incidence calculated on the basis of equal contribution by both parents.

‡ $^1/_2$ (0.94) + $^1/_2$(0.09) = 0.52.

§ $^1/_2$(0.94) + $^1/_2$(0.52) = 0.73.

‖ $^1/_2$(0.52) + $^1/_2$(0.09) = 0.31.

(*c*) each parent contributes equally to the genetic control of NTA production in the offspring (gene dosage).

Another genetic analysis is based upon the assumption that NZB mice carry a gene or genes that lead to 100% incidence of NTA (assuming modifying factors such as male sex hormones are eliminated). The model for this calculation (assuming complete penetrance) is shown in Fig. 1. The observed relative frequencies in gonadectomized animals are very close to, and not significantly different from, this theoretical model (Table III). However, in intact F_1 and backcross mice, the observed relative frequencies are significantly different from the expected theoretical relative frequencies. Thus, only the gonadectomized offspring (especially males) provided data compatible with the theoretical model.

IgM Concentration.—NTA are predominantly of the IgM class (3, 18). We therefore felt obliged to look for a possible correlation between NTA and IgM serum concentration in individual mice. In Fig. 2 the serum IgM concentrations for the two parental strains, the F_1 hybrids, and the backcrosses are shown. Overall, the amount of serum IgM increases as the contribution of the NZB genome increases. In the DBA/2 strain, the males have significantly less serum IgM than do the females; this sex difference wanes as the cross approaches the NZB genome. Thus, NZB males and females had similar IgM concentrations just as they had similar quantities of NTA. The gonadectomized males have higher IgM concentrations than intact males suggesting that male sex hormones nonspecifically decrease total serum IgM levels.

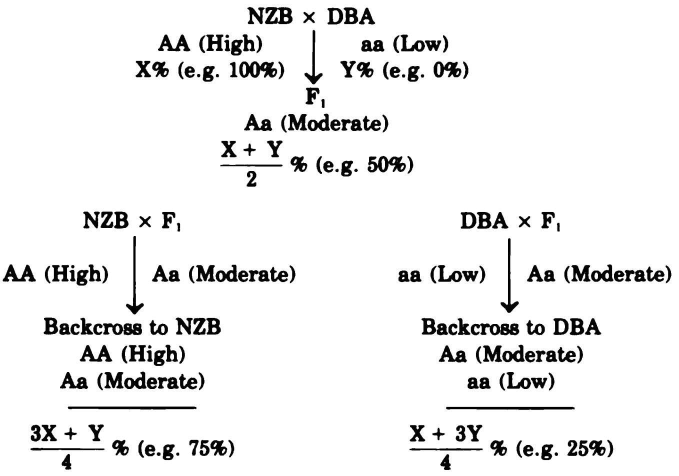
NZB × DBA
AA (High)
X% (e.g. 100%)
aa (Low)
Y% (e.g. 0%)
F_1
Aa (Moderate)
$\frac{X+Y}{2}$ % (e.g. 50%)
NZB × F_1
AA (High)
Aa (Moderate)
Backcross to NZB
AA (High)
Aa (Moderate)
$\frac{3X+Y}{4}$ % (e.g. 75%)
DBA × F_1
aa (Low)
Aa (Moderate)
Backcross to DBA
Aa (Moderate)
aa (Low)
$\frac{X+3Y}{4}$ % (e.g. 25%)

FIG. 1. Gene dosage model. Aa, moderate production; AA, high production.

TABLE III

Comparison of NTA Frequencies in Intact and Gonadectomized NZB, DBA/2, F_1, and Backcross Animals*

Animal	Expected‡	Intact		Gonadectomized	
		Observed	χ^2 (P)	Observed	χ^2 (P)
NZB	1.00	0.91	0.27 ($P > 0.50$)	0.94	0.06 ($P > 0.75$)
DBA/2	0.00	0.04	0.04 ($P > 0.75$)	0.09	0.18 ($P > 0.50$)
(NZB × DBA/2) F_1	0.50	0.24	14.52 ($P < 0.005$)	0.52	0.10 ($P > 0.75$)
F_1 × NZB	0.75	0.48	18.12 ($P < 0.005$)	0.71	0.15 ($P > 0.50$)
F_1 × DBA/2	0.25	0.09	7.33 ($P < 0.01$)	0.21	0.29 ($P > 0.50$)

* Frequencies based on calculations in Tables I and II.

‡ Based on theoretical gene model (Fig. 1).

The IgM levels followed a pattern similar to that of NTA: (*a*) both had decreased levels in males, (*b*) the DBA/2 strain was the lowest, (*c*) backcrosses to the DBA/2 were intermediate between the DBA/2 strain and the F_1 hybrids, and (*d*) the backcrosses to the NZB strain were intermediate between the F_1 hybrids and the NZB strain. In Fig. 3 A individual F_1 hybrid sera were analyzed for both the amount of serum IgM and the amount of thymocytotoxicity. Using Spearmann rank correlation analysis, no significant correlation was observed. There was also no correlation between IgM concentrations and thymocytotoxicity in backcrosses. Although both IgM levels and NTA are increased in NZB mice and their offspring, and in castrated males as compared with intact males, the increase in NTA is not the result solely of the increased IgM levels.

Spontaneously Produced ssDNA Antibodies. – In Fig. 4 the amount of ssDNA antibodies is shown for both parental strains, F_1 hybrids, and backcrosses. In

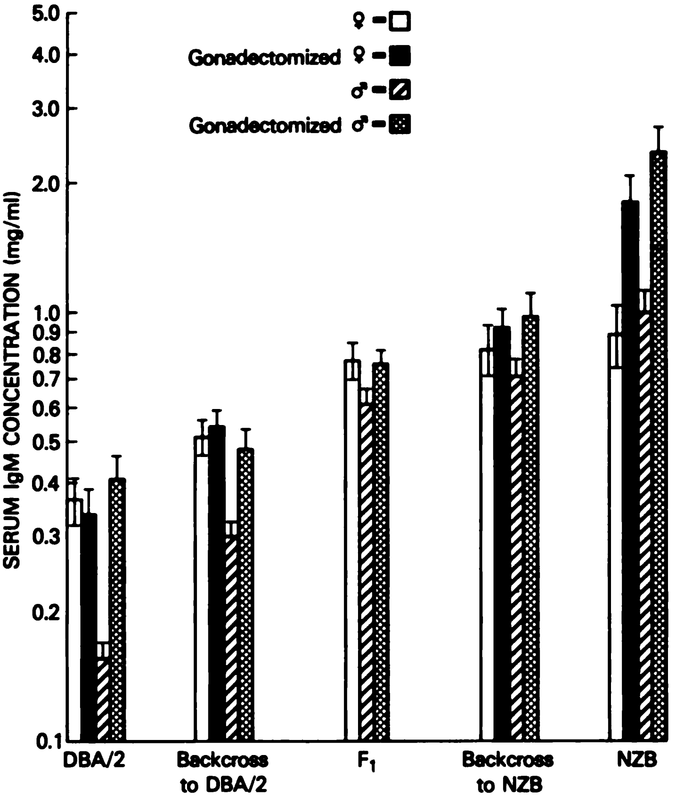

FIG. 2. Serum IgM concentrations were determined in intact and gonadectomized mice of both sexes. The means of 10 mice are shown in each column. Bars indicate the standard error of the mean. The difference between intact males and intact females was significant for DBA/2, and backcross to DBA/2 mice ($P < 0.05$). The difference between intact males and gonadectomized males was significant ($P < 0.05$) for all groups except for F_1 mice ($0.1 > P > 0.05$). Intact and gonadectomized females did not differ significantly except in the case of NZB mice ($P < 0.025$). Castrated males did not differ significantly from castrated females with regard to serum IgM concentrations.

all cases the intact males produced significantly less anti-ssDNA than did the gonadectomized males ($P \leq 0.05$, Student's *t* test). In all cases, except in the NZB strain, the females had more binding than the males. Gonadectomy of the males elevated their ssDNA binding to levels at least as high as the corresponding females. Analysis of the genetic contribution of the NZB parent to ssDNA antibodies is shown in Table IV. Unlike the case of NTA, groups of animals divided themselves very well into nonoverlapping positives and negatives. All NZB mice produced anti-ssDNA but none of the DBA/2 mice did, even when castrated (unpublished data). F_1 mice (both NZB and DBA/2 mothered) all produced anti-ssDNA providing males were castrated to reduce the "suppressive" influence of male sex hormones. Vasectomy and sham castration gave results similar to those found in intact males (unpublished data). Backcrosses to NZB mice gave a frequency close to 100%, and backcrosses to DBA/2 mice gave a frequency close to 50% (again providing males were castrated). These

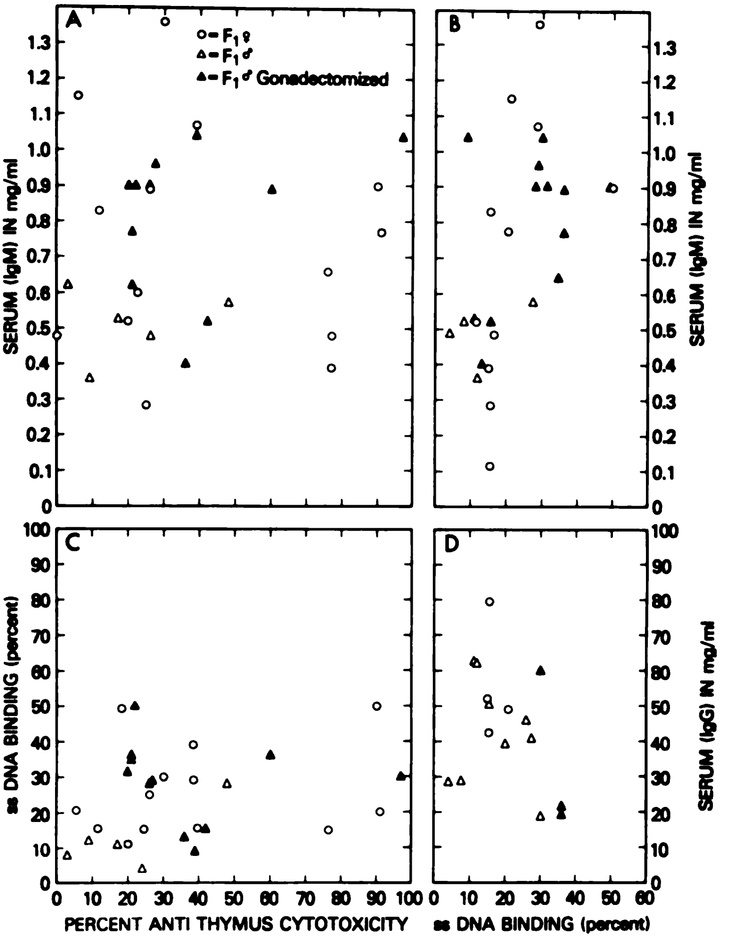

Fig. 3. (A) Serum IgM is plotted against NTA for individual sera. There was no significant correlation between the two measures (r_s = −0.2, $P > 0.06$). In addition, there was no significant correlation for subgroups (F_1 females, r_s = 0.03, $P > 0.05$; F_1 males, r_s = −0.10, $P > 0.05$; F_1 gonadectomized males r_s = 0.21, $P > 0.05$). (B) Serum IgM is plotted against antibodies to ssDNA. There was significant correlation between the two measures (r_s = 0.56, $P < 0.01$). (C) Antibodies to ssDNA are plotted against NTA in individual sera. No significant correlation was found between the quantities of the two antibodies for all sera (r_s = −0.12, $P > 0.05$). (D) Serum IgG is plotted against ssDNA binding. Overall there was no signficicant correlation (r_s = −0.36, $P > 0.05$).

data are best explained by a single dominant gene which predisposes to spontaneous anti-ssDNA production.

In anti-ssDNA-positive mice, the quantity of anti-ssDNA is not completely explained by a single dominant gene (Fig. 4). F_1, backcrosses to NZB, and NZB mice show a progressive increase in amount of anti-ssDNA, even though all have a 100% incidence of these antibodies. Quantitative analysis of the ssDNA binding suggests that the quantity of anti-ssDNA produced is controlled by a single regulatory gene. Complete data for castrated mice are available for males. Thus, DBA/2 male mice had close to 0%, F_1 mice 20%, and backcross to

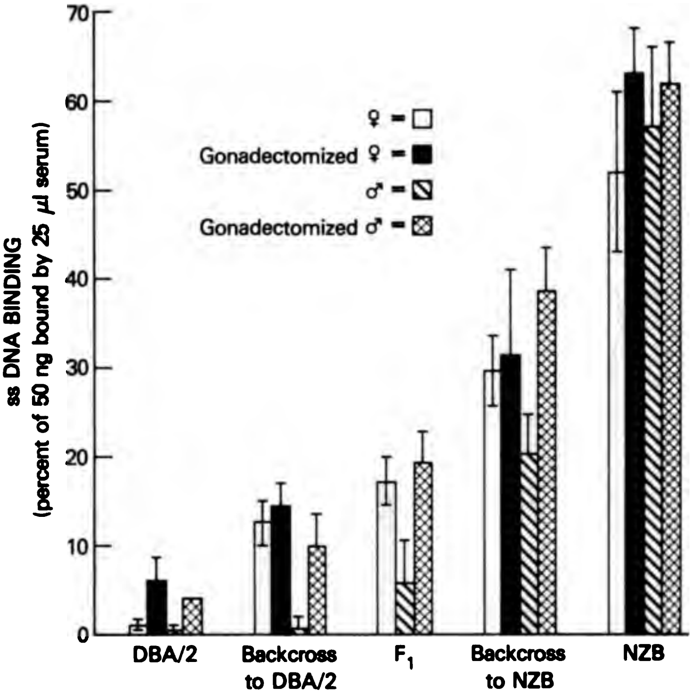

FIG. 4. Mean percent of 50 ng ^{14}C-labeled ssDNA bound by individual sera is shown in the vertical columns. Groups consisted of 10–28 mice. Bars represent the standard error of the mean. Intact females had significantly more anti-ssDNA than intact males in the F_1 mice and backcrosses to DBA/2 ($P < 0.05$). Gonadectomized males were significantly higher ($P < 0.05$) than intact males in all groups except the NZB mice. In all groups, castrated females and castrated males did not differ with regard to anti-ssDNA antibodies ($P > 0.05$).

DBA mice 10% ssDNA binding. NZB had close to 60%, and backcrosses to NZB had 40% binding (Fig. 4). This strongly suggests either (*a*) a gene dosage type of inheritance by a single-gene locus for anti-ssDNA (in Fig. 1, X ≃ 60%, Y ≃ 0%) of (*b*) one dominant and one regulatory gene as shown in Fig. 5. These two models cannot be distinguished by the data in Fig. 4.

Correlations of ssDNA Antibodies with NTA and Ig.—The NZB strain produces both NTA and antibodies reactive with ssDNA. To investigate the possible relationship between these two autoantibodies, individual sera were analyzed for the quantities of both NTA and ssDNA antibodies. Overall, there was no correlation between the percent cytotoxicity (NTA) and ssDNA binding. This is illustrated for F_1 mice in Fig. 3 C. Using Spearmann rank order analysis, there was no correlation between NTA levels and ssDNA antibodies in NZB, DBA, F_1, or backcrosses. By disregarding the quantity of autoantibody and considering only positive or negative expression of the trait, analyses can be performed in backcross mice to determine whether or not NTA and ssDNA antibody traits are linked. If the two genes segregate independently, there should be 50% of the parental types and 50% of the recombinant types. On the other hand, if the two loci are linked, the frequency of the recombinant types should be significantly lower than that of parental types (that is <50%). In

TABLE IV

Spontaneous Production of Antibodies against ssDNA in NZB, DBA/2, F_1, and Backcross Mice

Animal	Sex	Anti-ssDNA Positive		Expected*
		No./total	Frequency	
NZB	F	6/6	1.00	1.00
NZB	M	5/5	1.00	1.00
DBA/2	F	0/10	0.00	0.00
DBA/2	M	0/7	0.00	0.00
(DBA × NZB) F_1	F	13/13	1.00	1.00
(NZB × DBA) F_1	F	7/7	1.00	1.00
(DBA × NZB) F_1	M	12/12	1.00	1.00
(NZB × DBA) F_1	M	14/18	0.78	1.00
(NZB × DBA) F_1	M Castrated	7/7	1.00	1.00
F_1 × NZB	F	17/18	0.94	1.00
F_1 × NZB	M	14/19	0.74	1.00
F_1 × NZB	M Castrated	7/7	1.00	1.00
F_1 × DBA/2	F	14/28	0.50	0.50
F_1 × DBA/2	M	1/24	0.04	0.50
F_1 × DBA/2	M Castrated	5/15	0.33	0.50

* Expected frequency if propensity for anti-ssDNA antibody production is controlled by a single dominant gene.

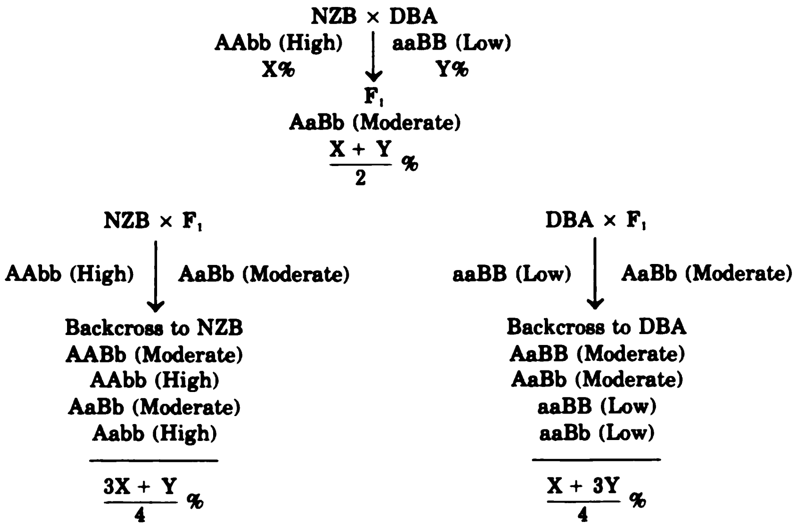
NZB × DBA
AAbb (High)
aaBB (Low)
X%
Y%
F_1
AaBb (Moderate)
$\frac{X+Y}{2}\%$
NZB × F_1
AAbb (High)
AaBb (Moderate)
Backcross to NZB
AABb (Moderate)
AAbb (High)
AaBb (Moderate)
Aabb (High)
$\frac{3X+Y}{4}\%$
DBA × F_1
aaBB (Low)
AaBb (Moderate)
Backcross to DBA
AaBB (Moderate)
AaBb (Moderate)
aaBB (Low)
aaBb (Low)
$\frac{X+3Y}{4}\%$

FIG. 5. Model for a dominant gene with a recessive modifier gene. A, dominant gene; b, recessive quantitative-modifier gene.

TABLE V
Distribution of NTA- and ssDNA-Positive and Negative Mice for All Backcrosses (to Both Parents)

	NTA +	NTA −
ssDNA +	26	44
ssDNA −	13	45

$\chi^2 = 1.32, P > 0.20$.

these experiments, parental types would be either positive for both NTA and anti-ssDNA or negative for both. Recombinant types would be positive for one trait and negative for the other. The observed ratios of the parental and recombinant types (Table V) do not differ significantly from the ratios expected if the two traits were not linked ($\chi^2 = 1.32, P > 0.20$). Furthermore, in the 28 female backcrosses to DBA/2, one-half of the mice produced anti-ssDNA and one-half did not (Table IV). In the 14 animals with anti-ssDNA, the mean percent thymocytotoxicity (NTA) at a 1:4 serum dilution was 8.71 ± 2.3 and in the 14 animals without anti-ssDNA the mean percent thymocytotoxicity was 14.38 ± 5.7. This further demonstrates that the genetic predisposition to the development of each of these two autoantibodies is inherited independently. Finally, because the amounts of these two autoantibodies are unrelated, it is likely that separate secondary (? regulatory) genes independently control the magnitude of the production of the two autoantibodies.

Serum IgG levels were not significantly correlated with the amount of ssDNA antibodies produced (Fig. 3 D). However, serum IgM levels were correlated with the amount of ssDNA antibodies (Fig. 3 B).

Discussion

An increasing body of evidence suggests that sex hormones influence spontaneous autoantibody production (19–21) and antibody response to immunization (22–25). Although female sex hormones may have some immunoregulatory effects, the predominant mechanism is an immunosuppressive effect of male sex hormones. The immunosuppressive effect is probably not related to direct suppression of B cells, but rather, indirectly, through an effect on thymocyte development and T cells (26). Because sex hormones may affect antibody production, it becomes important to consider the sex of study animals in genetic analyses of control of antibody production. This is especially relevant for situations in which males and females differ markedly. An extreme example of this phenomenon is the spontaneous production of NTA in NZB hybrids; male hybrids do not produce NTA, whereas many females do. Theoretically, one way to avoid this problem is to use only females. However, this is not possible when an X-linked immune response gene is under investigation. In such a situation, castration of progeny may allow for an adequate genetic analysis.

We found that only by looking at gonadectomized animals could a model be constructed for the genetic contribution of NZB parents to the appearance of NTA in offspring. A single locus in which there was a gene dosage effect could account for the observed results. If the NTA trait were dominant, all the F_1

hybrids would produce high titers of NTA, and if the trait were recessive none of the F_1 animals would produce NTA; neither was the case. In fact, 52% of the hybrids produced NTA. If a single gene for the phenotype is postulated, the F_1 hybrids would be heterozygous and could have only one gene predisposing to NTA, whereas the NZB strain has two genes. If there were a gene dosage effect, the F_1 hybrids would produce half as much NTA. Unfortunately, NTA production is variable in terms of time of onset and titer, even in the highly inbred NZB strain. As a result, the arbitrary selection of a positive test of 50% kill at a 1:4 serum dilution gave not a 100%, but approximately a 50% incidence level. It is possible that examination at a later time (e.g. 2 yr) would have allowed the F_1 mice to be closer to 100%. Unfortunately, at that age many NZB parents and backcrosses to NZB mice might already be dead of their autoimmune disease.

A model that explains these observations is shown in Fig. 1. The offspring receives an equal genetic contribution from each parental type for the phenotypic expression of the trait. However, each parental type has a distribution of the trait. Thus, an offspring has a probability of having the trait equal to the sum of each parent's probability divided by 2. The observed phenotypic expression in F_1 and backcross mice is very close to that expected whether calculated on the basis of observed expression (incomplete penetrance) in the parents (Table II) or an all-or-nothing expectancy (complete penetrance) in the parental strains (Table III). That both methods successfully approximate the observation shows the closeness of the phenotypic expression of the trait in NZB mice to 1.0 and in DBA/2 mice to 0.0.

Other alternative hypotheses are possible. The most plausible alternative is that NTA production is controlled by a single dominant gene but modified by an additional gene that controls the level of autoantibody production. One such model is shown in Fig. 5. Assuming that these two genes are not linked, an animal that is homozygous at both of these loci would produce maximal levels of NTA. If an animal were heterozygous at both loci, the recessive trait for excessive production of autoantibodies would not be expressed, but the dominant NTA trait would be expressed though not maximally. If an animal were heterozygous at the NTA locus but homozygous at the locus which controlled autoantibody levels, the production of NTA could be maximal. Either a gene dosage theory (Fig. 1) or a modifying gene theory (Fig. 5) would give the same expected results for NTA production. It is theoretically possible to distinguish a single gene (with a gene dosage effect) from a two-gene loci model (a dominant gene with a recessive modifying gene) by examining large numbers of F_2 mice. In the former, one would expect a 1:2:1 ratio, whereas in the latter one would expect a 4:9:3 ratio of low:moderate:high; however, in practice such an analysis might be extremely difficult because of the closeness of the two sets of ratios. Other genetic models cannot be excluded; however, the two presented here reasonably approximate the data.

The genetic analysis of the ssDNA antibody production suggested a single dominant gene that led to spontaneous anti-ssDNA production. The quantity of anti-ssDNA in positive mice is determined either by a gene dosage effect (Fig. 1) or a regulatory gene (Fig. 5). The genetic control of levels of ssDNA and NTA antibodies appear to be distinct.

In contrast, the level of ssDNA antibodies and quantity of IgM in individual mice were found to be correlated. This suggests that a regulatory gene model (Fig. 5) best explains the quantitative differences in anti-ssDNA levels among positive mice. In addition, these data suggest that the gene that regulates ssDNA antibody levels may be the same as (or linked to) the gene controlling the quantity of IgM.

It has been demonstrated in the present experiments that IgM levels of males are decreased relative to female littermates in intact DBA/2, backcrosses to DBA, and F_1 mice. This could not be strictly correlated with the decrease in NTA. Thus, the decrease in total IgM in intact males does not alone account for the decrease in this autoantibody. Male sex hormones may act to nonspecifically decrease total levels of IgM; they may work in another manner to prevent the formation of autoantibodies.

Large numbers of matings have been performed with NZB mice to determine the mode of inheritance of autoimmune traits. Two types of matings have been evaluated: (*a*) crosses with other New Zealand strains, and (*b*) crosses with non-New Zealand strains. In crosses of NZB with NZC mice, 100% of the F_1 animals were Coombs positive, and 74% of the backcrosses to NZC were Coombs positive, suggesting that a single gene was responsible for the abnormality (27). Additional studies of NZB X NZC mice have suggested both a dominant and recessive gene for antierythrocyte antibodies (28). Braverman (29) also studied Coombs positivity in crosses of NZB with NZW mice. He concluded that there is a dominant gene in NZB mice that determines Coombs positivity whereas the NZW carries "a modifying gene" that in the presence of the NZB gene allows for a positive antinuclear response. Thus, the non-NZB New Zealand strains contribute to the disease state observed in the backcrosses. It seems that the severity of the disorder in NZB crosses with other New Zealand strains depends on the particular New Zealand strain. With regard to antierythrocyte antibodies, the current view is that more than one gene, at least one of which may not be uniquely associated with the NZB strain, is involved in the phenotypic expression of these autoantibodies (30).

Additional studies of crosses with NZB and other New Zealand strains focused largely on questions of disease signs. Because disease may depend upon many factors, it may not be a good phenotype to study in genetic analysis. This is especially true of immune complex glomerulonephritis to which a variety of different antibodies appear to contribute. Despite these difficulties, the occurrence of lupus nephritis in NZB X NZW F_1 mice has been postulated to depend on the action of at least two dominant or codominant genes, at least one gene from each parent (31).

A variety of crosses of NZB with non-autoimmune, non-New Zealand strains produced F_1 hybrids with relatively mild autoimmune disease (32–35). Analysis of NZB X AKR F_1 mice and backcrosses to AKR parents suggested that three to five unlinked genes controlled the production of antierythrocyte autoantibodies and that one of the genes is on the X chromosome (32). In addition, autoimmune traits were found to be independent of xenotropic viral expression (36).

The main difficulty with all genetic analyses of inheritance of autoimmune traits is the complexity of the disease process. The production of disease signs and autoantibodies may be the end result of the action of several genes.

Autoantibody production, once considered manifestations of abnormal autoimmunocompetent lymphocytes, now are thought possibly to result from aberrant immunoregulation (37). Despite these problems, previous studies have suggested a limited number of genes controlling a variety of autoimmune phenomena in NZB offspring. We have confirmed and extended these suggestions in the present study. We have provided evidence for a single dominant gene controlling the expression of spontaneous anti-ssDNA antibodies. The quantity of anti-ssDNA produced is controlled either by gene dosage (i.e., two genes lead to more anti-ssDNA than one gene) or, more likely, by a second regulating gene. Spontaneous production of NTA appeared to be inherited as a single codominant trait. No linkage between the gene regulating NTA production and the genes regulating antibodies to ssDNA was found.

Inasmuch as both NZB and DBA/2 have the same H-2 type, the locus controlling production of either autoantibody appears not to be within the *H-2* complex. Other investigators have provided evidence for non-*H-2* immune response genes (15, 38–40). In addition to non-H-2 control of antigen recognition, there is non-H-2 genetic control of the amount of antibody synthesized (41, 42). For example, the control of anti-L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} is under H-2 control but the amount of antibody produced by responders is controlled by non-*H-2*-linked genes (43). At least one of the genes controlling the magnitude of the antibody response is linked to the immunoglogulin heavy chain allotype linkage group (43, 44). The possibility that the production of autoantibodies is linked to heavy chain allotype is under investigation.

Finally, the role of sex hormones in modifying disease has not been adequately considered in previous genetic analyses. In the present studies, it was of considerable interest that intact offspring did not provide clear-cut data with regard to the genetic contribution of NZB mice to autoantibody production in their offspring. However, when gonadectomized offspring were studied, the data suggested that a single codominant gene controlled the presence of NTA and that an independent dominant gene controlled the production of autoantibodies to ssDNA. The quantity of each autoantibody is determined either by gene dosage or an independent regulatory gene. In addition to these findings, this study provides evidence for the theoretical claim that genetic analyses of phenotypes modified by sex hormones are best analyzed in gonadectomized progeny.

Summary

The appearance of naturally occurring thymocytotoxic autoantibodies (NTA) and spontaneously produced antibodies to single-stranded DNA (ssDNA) was studied in NZB, and DBA/2 mice and their F_1 and backcross progeny. NTA production was markedly decreased in males; however, castrated males produced quantities of NTA similar to those of females. Because the amount of NTA was influenced by sex hormones, it was necessary to gonadectomize all progeny to determine the mode of inheritance. Such studies suggested that NTA production was determined by a single locus with a gene dosage (codominant) mode of expression.

The spontaneous production of antibodies to ssDNA appeared to be inherited as a single dominant genetic trait. The quantity of anti-ssDNA was also found

to be under additional regulation; either a gene dosage effect or more likely a regulatory gene. The genes controlling the presence and quantity of ssDNA antibodies were not linked to the gene controlling the appearance of NTA.

The authors are indebted to Dr. David Alling for helpful discussion and assistance with statistical analyses.

Received for publication 21 November 1977.

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SPONTANEOUS PYROGEN PRODUCTION BY MOUSE HISTIOCYTIC AND MYELOMONOCYTIC TUMOR CELL LINES IN VITRO*

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Fevers associated with neoplastic growth occur commonly in patients with acute leukemias (1), Hodgkin's disease (2), and other lymphomas (3), and less frequently in patients with carcinomas such as hypernephromas (4), hepatomas (5), or tumors metastatic to the liver (6). Since often no infectious agent can be implicated in such fevers, it is likely that many are caused by the malignant disease itself. Such tumor-associated fevers, like fevers studied in animal models, presumably result from the action of endogenous pyrogen (EP)[1], a small protein which is synthesized and released from leukocytes. When this protein enters the blood, it alters in some way the "set-point" of temperature-regulating neurons in the hypothalamus, inducing responses of heat production and conservation, and thus causing fever in the host (7). Experiments to date have shown that EP is only produced by granulocytes, monocytes, or tissue macrophages. In addition, these cells require stimulation by inflammatory agents such as endotoxin or phagocytosis before EP production and release occurs.

Fever associated with malignancies might be the result of EP production by neoplastic cells themselves. Alternatively, host mononuclear or polymorphonuclear leukocytes may be stimulated to release EP by factors released by tumor cells, or generated by other host cells in response to tumor growth. In previous studies, abnormal, spontaneous EP production was demonstrated in cell suspensions prepared from spleen and lymph nodes of patients with Hodgkin's disease (8), but the cell type(s) responsible for this production were not identified. We have now investigated release of EP by neoplastic cell lines in vitro, including six mouse tumor lines having the characteristics of leukemia or lymphoma cells, three carcinoma lines of human origin, and one viral-transformed mouse fibroblast line. Spontaneous EP release has been documented from five of the six mouse tumor cell lines.

Materials and Methods

All materials, glassware, and reagents were obtained or made sterile and pyrogen-free by methods described previously (9). All test supernates were centrifuged at 800–2,000 *g* for 10–15

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* Supported by research grants CA-14655 from the National Cancer Institute and AI-01564 from the National Institute of Allergy and Infectious Diseases.

[1] *Abbreviations used in this paper:* BCG, bacillus Calmette-Guérin; CSA, colony-stimulating activity; EP, endogenous pyrogen; LAF, lymphocyte-activating factor; LPS, lipopolysaccharide; MEM, Eagle's minimal essential medium; PF, pyrogen-free.

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min and cultured in thioglycollate broth before injection; any contaminated samples were discarded.

Pyrogen Assay. Culture supernates were tested for pyrogenicity using a mouse bioassay which has been described previously (10, 11). Briefly, 7–8-wk-old Swiss-Webster mice (Yale colony) were kept at 35°C for 1–2 h until stable temperatures were achieved. They were then injected intravenously with 0.3 ml of a test solution, usually a dilution of supernate from incubated cells, and rectal temperatures were monitored every 10 min for 50 min. Maximum temperature changes from baseline 20 min after injection were used to determine the pyrogen content of the sample.

Tissue Culture Cell Lines. All cell lines were maintained in 75 cm^2 plastic flasks (Falcon Plastics, Div. BioQuest, Oxnard, Calif.), and incubated in 5% CO_2, 95% air. Fresh medium was supplied every 2–3 days; for mouse tumor cell lines, only one-half the volume of supernate was replaced with fresh medium at each time interval.

Mouse tumor cell lines (J-774, PU5-1.8, WEHI-3, P388 D1, RAW-8, and R-8) were obtained from Dr. Peter Ralph, Walker Laboratory, Sloane-Kettering Institute for Cancer Research, Rye, N. Y. The characteristics and origins of these cells have been described previously (12–16). Briefly, J 774, PU5-1.8, and P388 D1 cells resemble macrophages or histiocytes; WEHI is a myelomonocytic leukemia cell line; RAW-8 and R-8 cells were derived from mouse lymphomas and resemble lymphocytes. Cells were maintained in RPMI-1640 medium (Grand Island Biological Co., Grand Island, N.Y.) with 10% heat-inactivated, filtered fetal calf serum, 50 U penicillin/ml and 50 μg streptomycin/ml. For cell transfer, $\cong$ 2 ml of supernate containing $\cong 10^4$ cells were transferred weekly to new incubation flasks with 30 ml fresh medium. Before transfer of J-774, PU5-1.8, or WEHI-3 cells, which grow as loosely adherent cell layers, cells were detached from the flask surface by vigorous pipetting.

For experiments with the histiocytic and myelomonocytic cell lines, flasks of a single line with nearly confluent, adherent cell layers were selected, usually 3–4 days after seeding. All supernatant medium was discarded, and flasks were rinsed four times with RPMI (without serum) warmed to 37°C. 10 ml RPMI with 2.5 mg/ml lactalbumin hydrolysate (ICN Nutritional Biochemicals, Div. International Chemical & Nuclear Corp., Cleveland, Ohio), pyrogen-free (PF) medium, were then added. In some experiments, 10% endotoxin free, heat-inactivated fetal bovine serum (Flow Laboratories, Inc., Rockville, Md.; lot 4055 1190) was used in place of lactalbumin. In other experiments, heat-killed staphylococci, prepared as described previously (11), were added to one of a pair of flasks at an estimated ratio of 10:1 bacteria to cells. The flasks were incubated for 18 or 42 h. For experiments with R-8 and RAW-8 cells which grow in suspension, 3–4 days after seeding the flask contents were removed, centrifuged at 100 *g* for 10 min, and the supernatant medium was discarded. The cells were resuspended in RPMI without serum, centrifuged at 100 *g* for 10 min, and this washing procedure was repeated three times. Cells were finally suspended in 10 ml PF medium and equal aliquots were incubated in new flasks for 18 or 42 h.

Human tumor cell lines (Caki-1, Caki-2, and SK-HEP-1) were obtained from Dr. Jörgen Fogh, also of Walker Laboratory. The characteristics and origins of these lines have been previously reported (17). Caki-1 and Caki-2 cells, derived from clear cell renal adenocarcinomas, were grown in McCoy's 5 A medium (modified) (Grand Island Biological Co.), containing 15% filtered fetal calf serum, 50 U penicillin/ml, and 50 μg streptomycin/ml. SK-HEP-1 cells, derived from an adenocarcinoma of the liver, were similarly maintained except that Eagle's minimal essential medium (MEM; Auto-POW, Flow Laboratories, Inc.) plus 2 mM L-glutamine was substituted for McCoy's medium. For transfer of cells, flasks with confluent layers were either treated with Viokase (11) (Grand Island Biological Co.) for Caki-1 and SK-HEP-1 lines, or cells were detached by scraping with a rubber policeman, for Caki-2 cells. One half of the cells recovered from each flask were transferred to new flasks containing 20 ml of the appropriate medium.

For experiments with human tumor cell lines, all supernate was removed from flasks with confluent cell layers, and the cells were rinsed three times with medium containing no serum. Flasks were then reincubated for 18 to 42 h with 5 ml of medium containing 15% pooled normal human serum. 5 ml medium with 15% human serum was also incubated as a control.

3T3-SV 40 mouse fibroblasts (Swiss-Webster origin) were obtained from B. Dorman, Department of Biology, and Dr. Paul Lebowitz, Department of Medicine, Yale University. Cells were grown in MEM (see above) with 2 mM L-glutamine, 10% fetal calf serum, 50 U penicillin, and 50 μg streptomycin/ml. Once a week, cells were transferred using Viokase as described above. For experiments, the same techniques were used for rinsing and incubating cell layers as those described for human tumor cell lines, except that 10% rather than 15% serum was used.

At the end of all incubations, supernates were removed, centrifuged at 800 or 2000 *g* for 20 min, and assayed for pyrogen content. Cells were also removed and counted, using a hemocytometer or Coulter counter (model Z_F; Coulter Electronics Inc., Hialeah, Fla.), and viability was measured in most experiments by exclusion of 1% eosin dye. When present, nonadherent cells were collected by initial centrifugation of the supernates at 100 *g*; adherent cells were obtained by scraping or by treatment with Viokase. For cells incubated with *Streptomyces albus*, percent phagocytosis was estimated from Wright's-stained coverslip smears. This varied from 59–77% in all experiments.

Heat and Pronase Treatment of Pyrogens. 50 mg of pronase (B-grade; Calbiochem, San Diego, Calif.) was dissolved in 5 ml saline, and preincubated for 2 h at 37°C. 0.05 ml was then added to 2.5 ml of a pyrogenic supernate and the mixture was incubated for 4 h at 37°C. Another 2.5 ml of the pyrogenic supernate was incubated for 3 h at 37°C, and then for 1 h at 56°C. A third aliquot was incubated for 4 h at 37°C with no additions. As a control, 2.5 ml tissue culture medium containing no pyrogen, with 0.05 ml pronase added, was also incubated at 37°C for 4 h.

Assays for Intracellular Pyrogen. Flasks of J-774 cells were chosen 3 or 4 days after cell transfer, and cells were incubated in PF medium for 18 or 42 h, as described above. Supernatant medium was removed for subsequent pyrogen assay, and cell layers were rinsed three times with warmed RPMI medium. 5 ml RPMI was then added to the flask, and the cells were detached with a rubber policeman. In some experiments, this cell suspension was transferred to a duplicate flask and additional cells were collected. Cell numbers in the suspensions, both total and percent viable, were determined as described above. The mixture was disrupted by five cycles of freeze-thawing in dry ice-acetone, centrifuged at 2,000 *g* for 20 min, and the supernate was then assayed for pyrogen content.

Sephadex Filtration of Pyrogenic Supernates. PF medium was added to flasks of J-774 cells as described previously, and after a 48-h incubation, it was removed and stored at −20°C. A pool of 100 ml medium was assayed for pyrogen content and then concentrated to 2.5 ml by evaporation through dialysis tubing in front of a fan. This material was filtered through a 48 × 2.5-cm Sephadex G-75 column, prepared as described previously (18) and equilibrated with phosphate-buffered saline, pH 7.4, containing 2.5 mg/ml lactalbumin hydrolysate. Sodium azide was omitted. 5-ml effluent fractions were collected, and 0.3-ml vol were injected for pyrogen assay. Three experiments with J-774 pyrogen were carried out, using two different columns. Human monocyte pyrogen was also prepared as described previously (18), and 1.5 ml containing four rabbit doses was filtered separately on one of the columns. Where pyrogen assays of individual tubes were carried out at several dilutions, a "derived" (ΔT) was calculated by taking the average values for peak ΔT and multiplying by the dilution. Markers to determine exclusion and filtration volumes for different molecular weights included blue dextran, ovalbumin (Worthington Biochemical Corp., Freehold, N. J.), and 2 × crystallized trypsin and ribonuclease A (both from ICN Nutritional Biochemicals). Protein markers were applied singly to each column after completion of the pyrogen studies using buffer containing no lactalbumin, and their filtration volumes were determined by measurement of optical density at 290 λ.

Results

Histiocytic and Myelomonocytic Tumor Cell Lines. Supernate from tissue culture flasks was obtained 24 or 48 h after incubation of the cells in PF culture media, and was tested in mice for pyrogenicity. The results of a representative experiment with J-774 cells are shown in Fig. 1. Although the supernate removed after 24 h of incubation was only equivocally pyrogenic, supernate from the 48-h incubation clearly produced a febrile response in mice. The shape of the fever curve, which peaked at 20 min after injection and rapidly returned to baseline or below, is identical to that produced by injection of mouse macrophage pyrogen, obtained after stimulation of normal peritoneal macrophages by phagocytosis (10, 11). Injection of media alone (control) caused no elevation of temperature. When results of a series of experiments were exam-

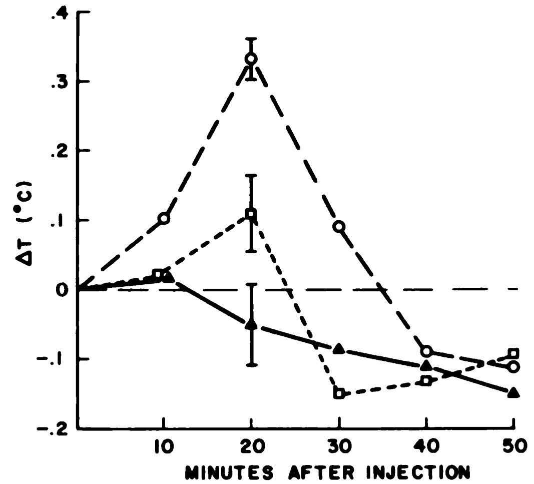

FIG. 1. Spontaneous pyrogen release by histiocytic tumor cells (J-774) during in vitro incubation. Average temperature change (ΔT) from baseline in groups of 11-12 mice after injection of culture supernates from 24-h (□) and 48-h (○) incubations of cells, or control media (▲), is shown. Each supernatant sample was derived from 1-5 × 10^5 viable cells. SEM at 20 min are shown by brackets.

ined, pyrogen was clearly detectable in medium from 24-h incubations, but larger amounts were always present after 48-h incubations. In addition, the same results were obtained when PF media contained fetal bovine serum in place of lactalbumin. These results suggest that J-774 cells spontaneously release pyrogen into the medium during in vitro culture.

Similar experiments were carried out with three other mouse cell lines: PU5-1.8 and P 388 D_1, which have the characteristics of histiocytic tumors, and WEHI-3, previously characterized as a myelomonocytic leukemia. In each instance, we obtained findings which were essentially identical to those described above for J-774 cells. A summary of our results is shown in Fig. 2. Pyrogen was present in supernates of 48-h incubations of all four cell lines. We initially tested the culture supernates in a wide range of doses, injecting media derived from 5 × 10^4-10^6 viable cells (complete data not shown). Since subsequent studies showed that maximal temperature elevations in the mice occurred regularly when media representing about 1-5 × 10^5 cells per dose was injected, this dilution of supernate was then tested most extensively for each cell line.

Lymphocytic Tumor Cell Lines. Spontaneous pyrogen release during in vitro culture was demonstrated in similar experiments using cells from one of two mouse cell lines previously characterized as closely resembling lymphocytic lymphoma cells (Fig. 3). Unlike the results with the macrophage lines discussed above, pyrogen in the PF culture medium of RAW-8 cells was more easily detectable after 24 h than after 48 h incubation. However, maximal fevers were produced as before by dosages of supernate corresponding to 1-5 × 10^5 cells/dose. We were unable to demonstrate any pyrogen release at either 24 or 48 h into PF media by the other lymphocytic tumor line, R-8, although doses of supernate corresponding to 10^5-10^6 viable cells were examined.

Effect of Phagocytic Stimulation on Pyrogen Release by Histiocytic Tumor

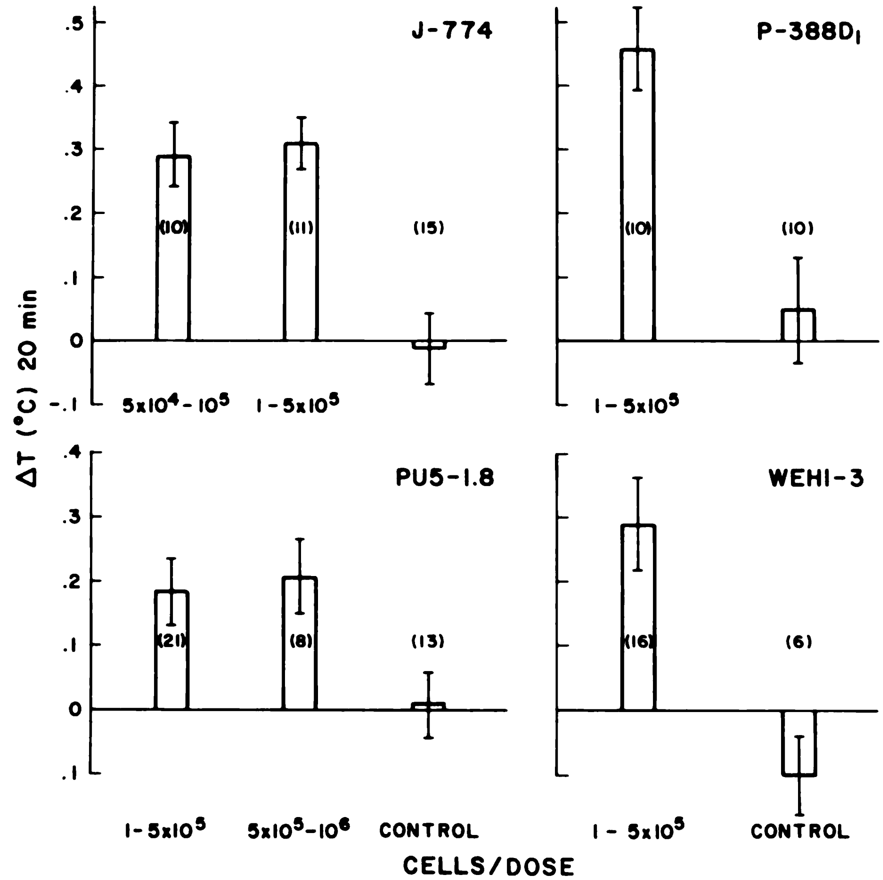

FIG. 2. Spontaneous pyrogen release by cells from three histiocytic tumor cell lines (J 774, P-388 D$_1$, PU5-1.8) and one myelomonocytic leukemia line (WEHI-3). In this and subsequent figures, average maximum temperature elevation (ΔT) ± SEM, 20 min after injection of culture supernates into groups of mice, is shown by the height of the bars, and numbers of mice in parentheses. Cells/dose, shown below the bars, indicates the number of viable cells recovered after incubation from which that dose of supernate was derived. In this experiment, cells were incubated in PF media for 48 h, and the supernate was diluted as needed and injected for assay. Control injections were of media incubated without cells.

Cells. We studied the effect of phagocytosis of staphylococci on pyrogen release by two histocytic cell lines, J-774 and PU5-1.8. In both cases, we noted increased fevers in the mice after injection of supernates from cells stimulated by ingestion of bacteria compared to the same numbers of cells releasing pyrogen spontaneously in culture (Fig. 4). Although the mouse assay for pyrogen is not quantitative over a large dose range, it is roughly quantitative over a two- to three-fold range between minimal and maximal temperature elevation, when increasing amounts of pyrogen are injected (10). These results, therefore, suggest that there were small, probably one- to twofold, increases in the amounts of pyrogen released by these cells after particle ingestion. We did not study either of the lymphocyte-like lines after incubation with staphylococci since we presumed they were nonphagocytic; R 8 cells do not ingest bacillus Calmette-Guérin (BCG) (19).

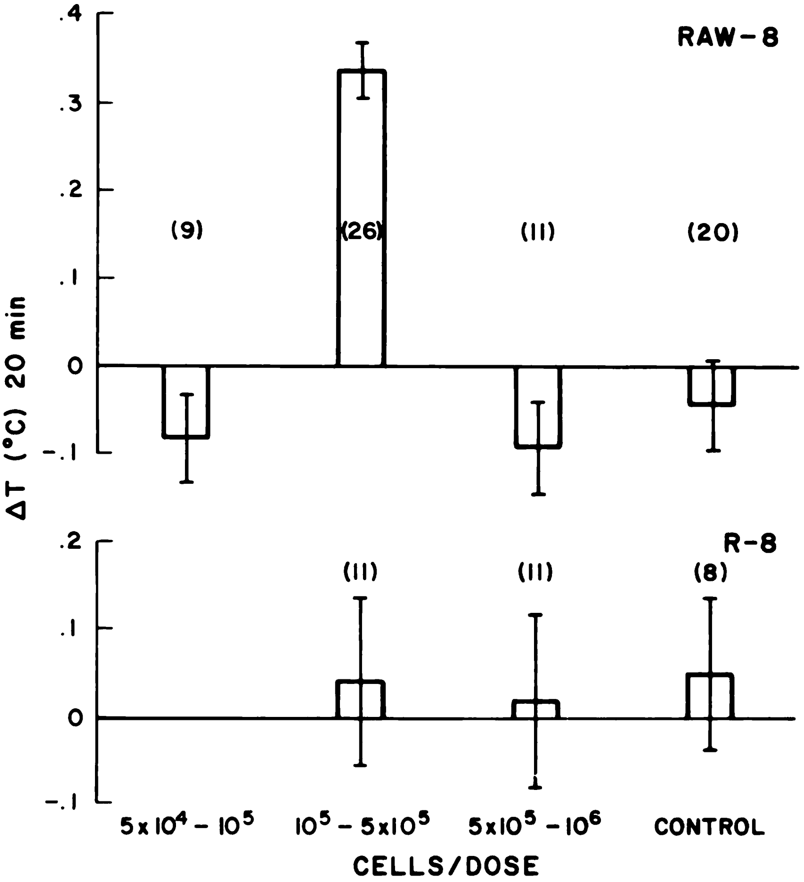

FIG. 3. Spontaneous pyrogen release by one of two lines of mouse lymphocytic-type tumor cells (RAW-8, R-8) during in vitro culture. Cells were incubated in PF media for 24 h before supernate was assayed. See Fig. 2 for other details.

Characteristics of Tumor Cell Pyrogens. As is illustrated in Fig. 1, the type of febrile response induced by the pyrogens from all five of these cell lines was identical to that produced in mice by normal leukocyte EP (10, 20). The pyrogens in flask supernates of the mouse tumor cells appeared to be more stable than those present in crude supernates of stimulated mouse macrophages, since they lost little activity during storage at −20°C for 1 wk, or −70°C for over 3 wk, whereas mouse macrophage pyrogen loses activity within days at 4°C and within 1 wk at −70°C (unpublished observations). However, the tumor cell pyrogens were inactivated by heating to 56°C for 1 h, and by incubation with pronase (Fig. 5). These characteristics suggest that they are proteins, like other endogenous pyrogens of leukocyte origin (7).

We obtained an estimate of the molecular weight of the pyrogen released by J-774 cells by filtration of a concentrate of culture supernate through a Sephadex G-75 column (Fig. 6). Two peaks of pyrogenic activity were present, one at a mol wt of ≅ 30,000, and the other at a mol wt ≥60,000, close to the exclusion volume of the column. Because the pyrogen assay in mice is not easy to

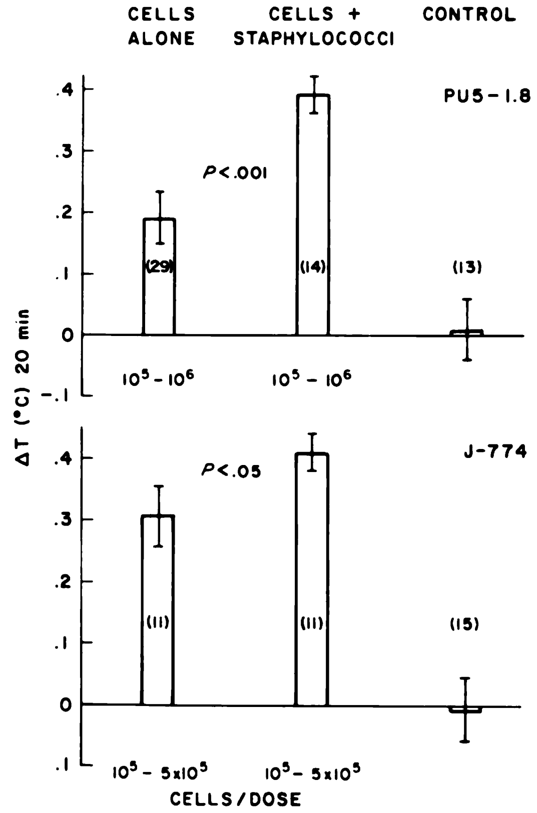

FIG. 4. Comparison of spontaneous pyrogen release by cells from two histiocytic tumor cell lines (PU5-1.8 and J-774) with that induced by phagocytosis of heat-killed staphylococci. Cells were incubated in PF media for 48 h after addition of bacteria or an equal volume of saline. The number of cells containing bacteria was estimated to be between 59 and 77% in all experiments. *P* values were calculated using Student's *t* test for small samples. See Fig. 2 for other details.

quantitate, it is not certain how much of the total pyrogenic activity was associated with each peak. However, the amounts appeared to be similar. By contrast, when supernate from human monocytes was applied to the same column, the two expected peaks of activity were noted, at mol wt of ≅ 14,000 and 40,000, and the quantity of the 14,000 mol wt pyrogenic species was clearly greater, as reported previously (18).

Mechanism of Production of Tumor Cell Pyrogen. We have done some preliminary studies to examine the mode of production of pyrogens by mouse tumor cells. When flasks containing J-774 or RAW-8 cells in PF media were placed at 4°C, supernates remained PF, in contrast to supernates of parallel flasks incubated as usual at 37°C. In addition, more pyrogen appeared in flasks of histocytic and myelomonocytic tumor cells as incubation continued for 48–72 h. Although we usually chose flasks containing moderate to large numbers of cells at 3, 4, or 5 days after seeding, pyrogen production appeared to progress during continued incubation regardless of the initial age of the culture. When rinsed cell layers were disrupted by freeze-thawing, and the resulting super-

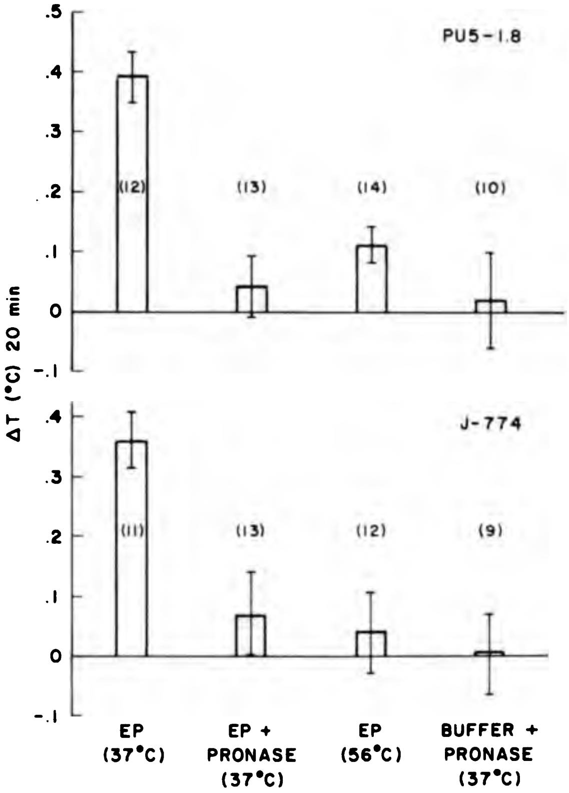

FIG. 5. Inactivation of pyrogenic supernates (EP) from two mouse histiocytic tumor cell lines (PU5-1.8 and J-774) by incubation with pronase or by heating for 1 h at 56°C. See text for details.

nates were assayed for progen content, at no time were significant amounts of intracellular pyrogen detected (Table I), even though pyrogen was present in the culture supernates of the same cells (positive assay results are underlined). The character of the PF medium in which cells were cultured to collect pyrogen for assay did not appear to affect pyrogen production, since we obtained identical results using medium containing either lactalbumin hydrolysate or a PF bovine serum. These findings, therefore, suggest that active production and secretion of pyrogen by these cells is occurring spontaneously and continuously during in vitro culture.

Comparison of Pyrogen Production with Other Characteristics of Mouse Tumor Cell Lines. As shown in Table II, pyrogen was produced by all tumor cell lines which possessed properties of macrophages, including the capacity for phagocytosis of bacterial particles or zymosan, presence of IgG receptors on the cell surface, and secretion of lysozyme. Although RAW-8 cells resemble lymphocytes morphologically, they secrete lysozyme in culture, and therefore presumably contain cells with macrophage characteristics (16). The cell line R-8 has not to date been reported to have any macrophage characteristics, and is presumably composed of lymphocyte-derived cells; cells of this line did not produce pyrogen.

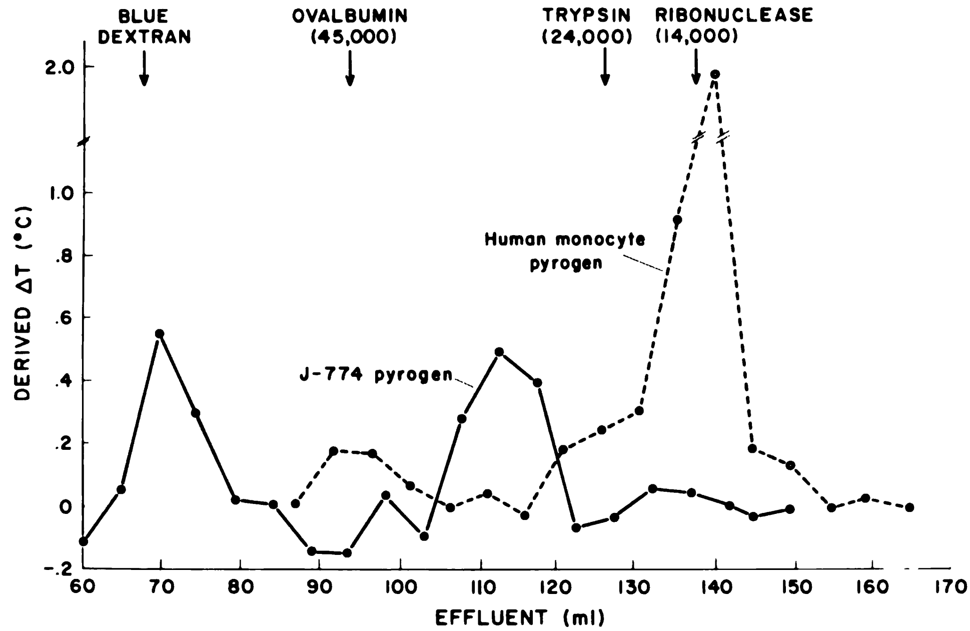

FIG. 6. Sephadex G-75 filtration of pyrogenic supernates from J-774 cells and stimulated human blood monocytes. 5-ml fractions were collected and 0.3-ml samples (undiluted or diluted 1:2-1:8) were injected into mice for pyrogen assay. Derived change in temperature (ΔT) was calculated as described in Materials and Methods. Each point represents the average temperature responses of 4-9 mice. Peak elution volumes of marker proteins are indicated by arrows.

TABLE I

*Differences in Intra- and Extracellular Pyrogen from J-774 Cells Incubated for Either 18 or 42 h**

	Cell incubation	Cells × 10^4/dose‡			Controls§
		5-10	11-49	49-100	
	h				
Supernate of disrupted cells	18	0.02 ± .07 (6)‖	−0.04 ± .05 (8)	−0.01 ± .06 (7)	
	42	−0.01 ± .05 (7)	0.00 ± .05 (15)	−0.10 ± .03 (11)	
Culture supernate	18	ND¶	0.18 ± .04 (10)	ND	−0.11 ± .03 (32)
	42	ND	0.29 ± .03 (23)	ND	

* Average change in temperature from baseline 20 min after injection, ± SEM.

‡ Cells from which supernate was derived for injection.

§ Injection of medium alone.

‖ Number of injections.

¶ ND, not determined.

Studies of Pyrogen Release by Human Carcinoma Tumor Cell Lines and Viral-Transformed Mouse Fibroblasts. Since patients with tumors other than leukemias and lymphomas occasionally suffer from prolonged, unexplained fevers, we also studied the capacity for pyrogen production by cells from three tumor cell lines derived from human carcinomas, two renal cell carcinomas (CAKI-1 and CAKI-2), and one hepatoma (SK-HEP-1). In addition, we tested

TABLE II
Mouse Tumor Cell Lines

Cell line	Origin	Morphologic and growth characteristics	Functional properties	Lysozyme secretion	LAF secretion (LPS added)	Pyrogen secretion	
						Spontaneous	After phagocytosis
J774	Balb/c derived from reticulum cell sarcoma, ascites form (13, 19)	Well-differentiated histiocytic lymphoma; loosely adherent (21)	Phagocytic: BCG, zymosan, latex, ab-coated erythrocytes, staph; ab-dependent target cell lysis (19, 21)	++ (16, 19)	++ (24)	++	+++
PU5-1.8	Balb/c derived from lymphoma PU 5-1, ascites form (16)	Histiocytic, loosely adherent	Phagocytic: BCG, zymosan, latex, staph; produces CSA after LPS (25)	++ (16, 19)	+ (24)	+	++
P388D1	DBA/2 derived from methylcholanthrene-induced lymphoid tumor, ascites form (12)	Histiocytic, loosely adherent	Phagocytic: BCG, latex, zymosan, staph; surface C_3 and F_c IgG receptors; "Nonspecific" esterase +; lyses ab-coated erythrocytes (12, 19)	++ (16)	++ (24)	++	ND
WEHI-3	Balb/c cloned from a myelomonocytic leukemia (14)	Monocytic, loosely adherent, differentiates into granulocytic and monocytic colonies (22)	Phagocytic: BCG, zymosan, latex; produces CSA (19, 22)	+ (19)	+ (24)	++	ND
RAW8	Balb/c derived from Abelson leukemia virus-induced lymphoma (15, 23)	Lymphocytic; nonadherent		+ (16)	ND[*]	+	ND
R8	Balb/c × C 57 BL/6 derived from Abelson virus-induced lymphosarcoma, "B" lymphoma (15)	Lymphocytic; nonadherent	Not phagocytic for BCG (19)	– (16)	ND	–	ND

[*] ND, not determined.

the hypothesis that viral transformation might confer the potential for pyrogen production on a mouse fibroblast cell line previously shown not to produce pyrogen spontaneously (11). We therefore tested incubation media obtained after 18- and 42-h incubations from the three human carcinoma cell lines, and after 18-h incubations from 3T3-SV 40 transformed mouse fibroblasts. We injected dosages of supernate derived from 0.5–49 × 10^4 cells for CAKI-1 and -2 lines; 1->50 × 10^4 cells for the SK-HEP-1 cells, and 5->100 × 10^4 cells for the mouse fibroblasts. 6–20 mice were used for each assay. In no instance were we able to demonstrate spontaneous pyrogen release by any of these cell lines. Although the carcinoma cell lines were of human origin, we have shown elsewhere (20) that pyrogen released by human leukocytes is easily detectable in mice.

Discussion

Unexplained fevers occur commonly in diseases such as acute leukemias, Hodgkin's disease, and other lymphomas (1–3), most of which are characterized by proliferation of phagocytic cells of bone marrow origin, including granulocytes, monocytes, and macrophages or histiocytes. Normal cells of these types produce pyrogen only after stimulation with an exogenous agent, such as endotoxin or phagocytosis, and production of pyrogen ceases after 1-2 days (7). In the studies reported here, we have demonstrated that three mouse tumor cell lines with characteristics of macrophage or histiocytic tumors, and one identified as a myelomonocytic leukemia, release pyrogen during in vitro culture. Moreover, this release appears to occur spontaneously and continuously during cell growth and multiplication. This conclusion is supported by our data showing that increasing amounts of pyrogen relative to cell numbers appear in the culture media with time, that similar amounts of pyrogen are produced by cells chosen either 3, 4, or 5 days after seeding, and that pyrogen accumulates in culture media which is identical to standard growth media except for the absence of endotoxin. Thus, these tumor cells appear to have retained the capability to produce pyrogen that is characteristic of their nonmalignant cells of origin, but to have acquired an altered mechanism for production of pyrogen, since they apparently require no initiating stimulus, and production does not cease after 24-48 h.

In preliminary studies, we have determined that production and secretion of the tumor cell pyrogens is an active, energy-requiring process. Cells kept at 4°C failed to release pyrogen. At no time were we able to detect significant levels of intracellular pyrogen in the one cell line examined, even though pyrogen was present in increasing amounts in the extracellular medium. Small but significant increases in pyrogen production occurred after incubation of cells from two histiocytic tumor lines with staphylococci, during which time most cells ingested bacteria. These results are similar to those obtained in studies of pyrogen synthesis and release by normal granulocytes and monocytes (7), indicating that the tumor cells retain some of the properties associated with pyrogen production of their cells of origin. On the other hand, as noted above, they appear to have lost at least a part of the normal control mechanisms for initiation and cessation of transcription of the pyrogen molecule, presumably a gene-mediated function (26, 27).

The pyrogens released by these tumor cell lines appear similar to the endogenous pyrogens that are produced when normal phagocytic cells of bone marrow origin are stimulated by phagocytosis or other inflammatory agents. Pyrogen produced by the macrophage tumor lines J-774 and PU5-1.8 were inactivated by heating at 56°C for 1 h and by a 4-h incubation with pronase. All pyrogens produced febrile responses characteristic of EP in mice, as illustrated in Fig. 1. These properties are the same as those of endogenous pyrogens derived from normal human (20), rabbit (P. Bodel, unpublished observations) and mouse (10) granulocytes, monocytes, or macrophages. The estimated molecular weights of pyrogens produced by J-774 cells were, however, larger than those reported for all other endogenous pyrogens previously studied, including those produced by granulocytes, monocytes, and macrophages of human or rabbit origin (18, 28-30). We have not yet determined the molecular weight of mouse macrophage pyrogen because of the lability of the molecule; possibly, pyrogens from mouse leukocytes will also prove to be of unusual size. Alternatively, pyrogenic molecules produced by tumor cells may have different physico-chemical characteristics from pyrogens produced by their nonmalignant cells of origin.

When tumor lines of carcinomas or viral-transformed fibroblasts were studied, we failed to detect any pyrogen in culture supernates. Thus, although many carcinomas are associated with unexplained fever in occasional patients, our studies do not provide support for the hypothesis that such tumor cells can produce pyrogen. These data extend our previous observations that HeLa cells and fibroblasts did not produce pyrogen, even after ingestion of particles (11). Possibly, fever in patients that is associated with the growth of carcinomas is a result of host cell interactions with the tumor, producing stimulation of host granulocytes, monocytes, or macrophages to produce pyrogen, rather than the result of the release of pyrogen by the tumor cells themselves.

The same cell lines that released pyrogen in our studies have been shown by others to secrete lysozyme, and in all but one instance (RAW-8), to have other macrophage characteristics, detailed in Table II. Two of the cell lines produce colony-stimulating activity (CSA), either spontaneously (19), or after addition of agents such as lipopolysaccharide (LPS) or zymosan (25). Lymphocyte-activating factor (LAF) is produced by all four macrophage tumor cell lines, after stimulation by LPS (24). It is noteworthy that tumor cells of presumed B-lymphocyte origin (R-8) did not produce pyrogen in our experiments. Normal lymphocytes have never been found to produce pyrogen, even after antigenic or blastogenic stimulation (7).

Our results raise the possibility that tumor-associated fever in patients with certain malignancies, such as some acute leukemias and lymphomas, may be due to the production of endogenous pyrogen by the malignant cells of granulocytic or monocytic origin. By extrapolation from studies of the cell lines WEHI-3 and RAW-8, it seems likely that the pyrogen molecules can be produced by immature cells at a stage of maturation preceding differentiation into granulocytes or monocytes. The hypothesis that malignant granulocytes and macrophages release pyrogen spontaneously during growth would be consistent with the observation that tumor-associated fever occurs in patients with these diseases during relapse, or at times of disease activity, but not during remissions (1–3). Preliminary studies have also shown that the human histiocytic tumor cell line, U-937 (16), also releases pyrogen during in vitro culture (P. Bodel and P. Ralph, unpublished observations). Such cells may provide an easily available source of human EP for laboratory investigations.

Summary

Tumor-associated fever occurs commonly in acute leukemias and lymphomas. We investigated the capacity for in vitro production of pyrogen by three mouse histiocytic lymphoma cell lines (J-774, PU5-1.8, p 388 D_1), one myelomonocytic line (WEHI-3), and two lymphoma-derived lines, RAW-8 and R-8. Pyrogen was released spontaneously into the culture medium during growth by all cell lines with macrophage or myeloid characteristics including lysozyme production; R-8 cells, of presumed B-lymphocyte origin, did not produce pyrogen. When injected into mice, the pyrogens gave fever curves typical of endogenous pyrogen, were inactivated by heating to 56°C and by pronase digestion, and appeared to be secreted continuously by viable cells. Two pyrogenic molecular species produced by J-774 cells were identified by Sephadex filtration, one of mol wt $\cong$ 30,000,

and the other ≥60,000. By contrast, three carcinoma cell lines of human origin and SV-40 3T3 mouse fibroblasts did not produce pyrogen in vitro. These results suggest that some malignant cells derived from phagocytic cells of bone marrow origin retain their capacity for pyrogen production, and may spontaneously secrete pyrogen during growth.

The author thanks Ms. Heather Miller for carrying out the reported work on human cell lines and fibroblasts, and Doctors Elisha Atkins and Robert M. Donaldson for their helpful criticism during preparation of the manuscript.

Received for publication 9 January 1978.

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GENETIC CONTROL OF THE IMMUNE RESPONSE TO STAPHYLOCOCCAL NUCLEASE

VIII. Mapping of Genes for Antibodies to Different Antigenic Regions of Nuclease

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Staphylococcal nuclease is a well-characterized protein antigen that has been used in our laboratory for a variety of studies on the genetic control of immune responses (1). Rat antisera detecting binding site-specific idiotypic determinants of anti-nuclease antibodies have been used to study the genetics of variable region genes (2–4). Thus, genes determining the predominant idiotype produced in A/J mice in response to nuclease were found to be linked to the heavy chain allotype locus, were subject to recombination with respect to this locus at a high frequency, and were independent of *H-2*-linked *Ir* genes in their expression (3, 4). In a preliminary study (3), it was shown that there existed at least two distinct noncross-reacting idiotypes determined by genes linked to heavy chain allotype linkage group (IgCH)[1] characteristic of strain A/J ($IgCH^e$) or SJL ($IgCH^b$). These two idiotypes were both present in immune sera from strain BALB/c ($IgCH^a$), but they were absent from sera of strain CB.20 ($IgCH^b$). The strain BAB.14 ($IgCH^b$), a recombinant strain which was produced during the transfer of the $IgCH^b$ allotype locus of B6 to the BALB/c background, bore only the A/J idiotype. This result suggested that the recombinational event in the BAB.14 occurred within the gene segment which codes for variable regions of the immunoglobulin heavy chain (V_H) rather than between constant region (C_H) and V_H genes as had been inferred from other studies (5–7).

A genetic map of V_H region genes in the BALB/c strain was proposed as:

$$A/J\alpha Nase - SJL\alpha Nase - IgCH^a$$

where A/JαNase and SJLαNase represent loci for genes determining A/J and SJL anti-nuclease idiotypes, respectively, and $IgCH^a$ represents the heavy chain allotype linkage group of BALB/c (8). From these studies, however, it was not possible to determine whether the A/J and SJL idiotypes were directed against the same or different antigenic determinants, or whether in fact they were on the same or different molecules. To answer some of these questions, anti-nuclease antibodies from various strains have been fractionated into two populations on the basis of their ability to bind to a fragment of nuclease comprising the 99th–149th amino acids. By analysis of the idiotypes in these two fractions, the map order for strain BALB/c has been confirmed, and at least three new idiotypic markers have been identified, allowing additional map positions to be assigned. These results and analyses are the subject of this report.

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[1] *Abbreviations used in this paper*: BSA, bovine serum albumin; CFA, complete Freund's adjuvant; C_H, constant region of the immunoglobulin heavy chain; CNBr, cyanogen bromide; IgCH, heavy chain allotype linkage group; V_H, variable region of the immunoglobulin heavy chain.

Materials and Methods

Mice. A/J mice were purchased from The Jackson Laboratory, Bar Harbor, Maine; SJL mice from Texas Inbred Mice Co., Houston, Texas; and BALB/c from National Institutes of Health Animal Production. CB.20 mice were a gift of Dr. M. Potter of the National Cancer Institute. BAB.14 mice were a gift of Dr. M. Weigert, Institute for Cancer Research, Philadelphia, Pennsylvania. All mice were males except those of strain SJL.

Rats. Adult male Lewis rats were purchased from Microbiological Associates, Walkersville, Md.

Preparation of Anti-Nuclease Antibodies. Groups of 5–25 mice were immunized with 100 μg of purified nuclease in complete Freund's adjuvant (CFA) and boosted weekly with 10 μg of nuclease in saline beginning on the 3rd wk after immunization. For the experiments reported here, pooled hyperimmune sera obtained after the 4th and 5th boost were used.

Preparation of Anti-Idiotypic Antisera. Lewis rats were immunized every 2 wk with 500 μg of affinity column-purified anti-nuclease antibodies in CFA. Sera from individual animals showing the highest activity were used in these experiments.

Assay of Anti-Nuclease Antibodies. The assay of anti-nuclease antibodies has been previously described (9). Briefly, the ability of anti-nuclease antibodies to inactivate the enzymatic activity of nuclease was determined at increasing dilutions of serum, and the plateau value for units inactivated per milliliter of antiserum was calculated.

Assay for the Presence of Idiotypes in Anti-Nuclease Antibodies. The details of this assay have been previously reported (2). Briefly, the ability of anti-idiotypic antisera to inhibit the inactivation of nuclease by antibodies was determined. To perform the assay, an anti-nuclease preparation was preincubated with anti-idiotypic antiserum for 5 min, and then incubated with nuclease. The units inactivated were determined and then compared to an identical amount of anti-nuclease antibody preincubated with 0.1% bovine serum albumin (BSA) in saline. The inhibitory activity of the anti-idiotypic antisera was calculated using the formula

$$\%\ \text{inhibition} = \frac{A(\text{Nase} + \alpha\text{Nase} + \alpha\text{ID}) - A(\text{Nase} + \alpha\text{Nase})}{A(\text{Nase}) - A(\text{Nase} + \alpha\text{Nase})}$$

in which $A(\text{Nase} + \alpha\text{Nase} + \alpha\text{ID})$ indicates the activity measured in the presence of nuclease (Nase), antibody (αNase), and anti-idiotype (αID), etc., as previously described. In all cases, the final dilutions of the antibody preparations used had comparable activities in terms of units of nuclease inactivated. Results presented were obtained from at least duplicate determinations on each antibody preparation. An antibody preparation was considered to contain a given idiotype if the anti-idiotype produced statistically significant ($P < 0.05$) inhibition of inactivation.

Preparation of Nuclease Fragment (99-149). The nuclease fragment comprising the carboxy terminal 99th-149th amino acids was prepared according to published methods (10). Briefly, nuclease was degraded with cyanogen bromide (CNBr) and the resulting cleavage products were separated by gel filtration chromatography on Sephadex G-50. The major peak with absorbance at 280 nm was then chromatographed twice on phosphocellulose, developing the column with a linear elution gradient of ammonium acetate 0.1 M, pH 3, to 1.0 M, pH 8. Purity of the resulting peptide was assessed by use of a complementation assay in which the ability of nuclease fragment (99-149) to combine with nuclease fragment (1-126) to form an enzymatically active structure was determined (11). By comparison with a preparation of fragment (99-149) of known purity, a purity of $\cong$90% was determined for the preparation used in these experiments.

Fractionation of Anti-Nuclease Antibodies. To separate antibodies capable of binding to the fragment (99-149), affinity chromatography was employed. Fragment (99-149) was coupled to

TABLE I

Strains Used in the Present Study

Strain	*H-2* Haplotype	Allotype	Anti-nuclease idiotypes
A/J	*a*	*e*	A/J
SJL	*s*	*b*	SJL
B10.A	*a*	*b*	B10
BALB/c	*d*	*a*	A/J, SJL
CB.20	*d*	*b*	B10
BAB.14	*d*	*b*	A/J

CNBr-activated Sepharose 4B as previously described (12). Hyperimmune sera were passed over this column. The antibodies not binding were considered to be devoid of specificities for determinants represented on the fragment and were termed anti-nuclease $(1\text{-}99)_N$, in which the subscript N indicates that the antibodies were prepared against intact, native nuclease, according to previously established conventions (13). To remove bound antibodies, the column was eluted with 0.5 M guanidine, 0.01 M KPO_4, pH 7, containing 0.1% BSA. The material obtained by this elution method was termed anti-nuclease $(99\text{-}149)_N$. Fractionated antibody preparations could be stored at −20°F without loss of activity for at least 2 mo.

Results

Fractionation of Antibodies. The fractionation procedure used in these experiments is based on the ability of antibodies prepared against the native form of a protein antigen to bind to a peptide fragment of the protein. This approach has been previously used in fractionation of goat anti-nuclease antibodies to yield preparations of restricted heterogeneity (12), and a theoretical basis for this fractionation has been described in terms of the conformational equilibrium of the peptide in solution (14). The peptide used in these studies, nuclease (99-149), comprises the carboxy terminal 99th to 149th amino acids of nuclease.

Table I lists the strains used in these studies along with their *H-2* haplotype, C_H allotype, and known anti-nuclease idiotypes. Antibodies from these strains were fractionated into anti-nuclease $(99\text{-}149)_N$ and anti-nuclease $(1\text{-}99)_N$ fractions as described in Materials and Methods. Various eluting conditions were tested for their ability to remove bound antibodies from the column; the most stable and active preparations were obtained using 0.5 M guanidine containing 0.1% BSA. Higher concentrations of guanidine or other chaotropic agents were able to remove slightly more bound protein, but the resulting preparations were less active. With the elution conditions described, the anti-nuclease $(99\text{-}149)_N$ fraction contained from 2 to 6% of the inactivating capacity of the unfractionated sera for the strains examined. In contrast, anti-nuclease $(99\text{-}149)_N$ preparations from goat anti-nuclease antisera comprised 11.5% of the total antibodies (12). It has not yet been determined whether in mice anti-nuclease $(99\text{-}149)_N$ antibodies represent a smaller percentage of the total anti-nuclease response than in the goat, or whether the mouse antibodies have become denatured during the fractionation procedures yielding preparations of lower specific activity.

Idiotype Analysis of Antibody Preparations. The idiotypes present in fractionated and unfractionated antibodies of the five strains tested were assayed by use of antisera prepared in Lewis rats against purified anti-nuclease

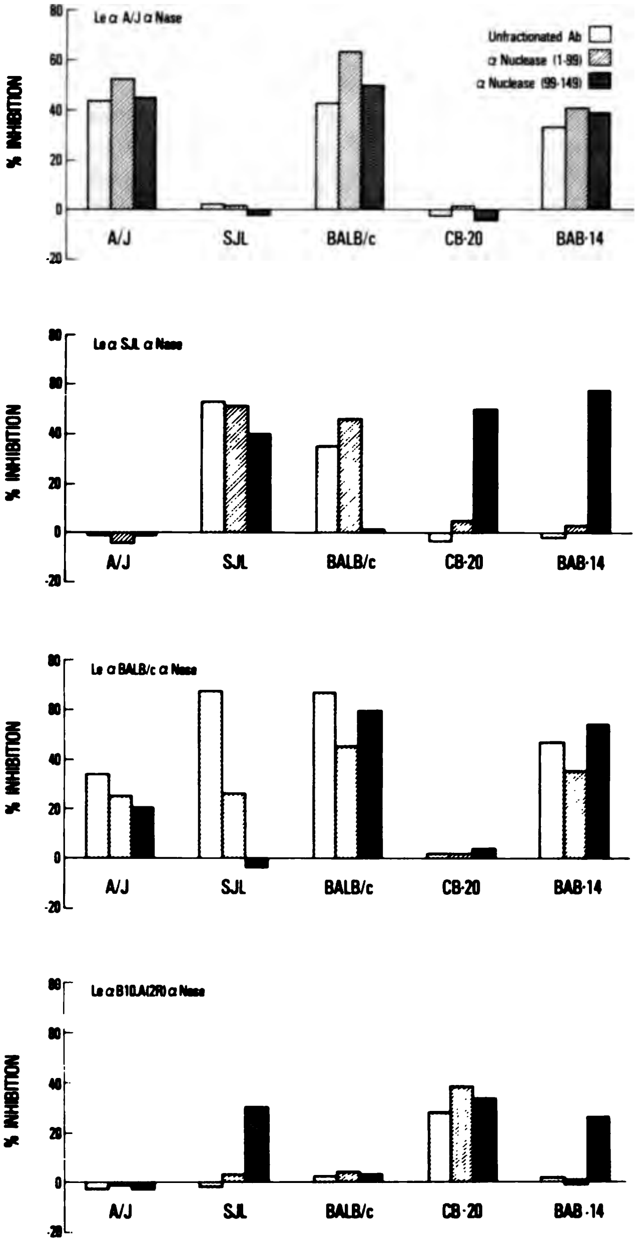

FIG. 1. Determination of anti-nuclease idiotypes by inhibition of antibody inactivation. Anti-nuclease antibodies from strains A/J, SJL, BALB/c, CB.20, and BAB.14 were fractionated into anti-nuclease $(1\text{-}99)_N$ and anti-nuclease $(99\text{-}149)_N$ as described in Materials and Methods. Fractionated antibody preparations as well as unfractionated antisera were tested with anti-idiotypic antisera specific for A/J, SJL, BALB/c, or B10.A(2R) anti-nuclease antibodies. Results are plotted in terms of the percentage of antibody-mediated inactivation that is inhibited by the anti-idiotypic antisera.

antibodies from strains A/J, SJL, BALB/c, and B10.A(2R). B10.A(2R) is a high responder strain with a recombinant *H-2* haplotype on the B10 background. Since *H-2* type has been found not to influence idiotype expression (3), the markers recognized by this sera will be referred to (for simplicity) as B10 idiotypes.

Fig. 1 presents in graphic form the idiotype analysis of the unfractionated antibodies as well as the anti-nuclease (1-99)$_N$ and anti-nuclease (99-149)$_N$ preparations from the strains analyzed. The percent inhibition of inactivation produced by the different idiotypes is shown. It is apparent from this figure that each anti-idiotypic antiserum was capable of recognizing at least two sets of idiotypic determinants, one detected in the anti-nuclease (1-99)$_N$ population and the other in the anti-nuclease (99-149)$_N$ populations. Thus, additional anti-nuclease idiotypes can be defined and distinguished in terms of the specificity of the antibodies, i.e. either anti-nuclease (1-99)$_N$ or anti-nuclease (99-149)$_N$.

Also apparent in Fig. 1 is the fact that in all cases, the pattern of reactivity of the anti-nuclease (1-99)$_N$ fraction was the same as that of unfractionated antibodies. This result was interpreted to indicate that the inhibition of nuclease inactivation assay for idiotypes detects only those idiotypes present in highest concentration. Studies by Berzofsky et al. (15) showed that within the anti-nuclease sera of strains A/J, SJL, and B10.A, antibodies with specificity for fragment (99-149) were less abundant than antibodies with specificity for fragment (1-126). Since anti-nuclease (1-99)$_N$ should be of similar composition to anti-nuclease (1-126)$_N$, this result is consistent with our observation that idiotypes of anti-nuclease (1-99)$_N$ were more readily detected in unfractionated antisera than idiotypes of anti-nuclease (99-149)$_N$.

The most surprising and, perhaps, important result seen in Fig. 1 was that the pattern of reactivity with the anti-idiotypic antisera of the anti-nuclease (99-149)$_N$ and anti-nuclease (1-99)$_N$ antibody populations differed for several strains. The following differences were noted. (*a*) Anti-nuclease (1-99)$_N$ antibodies from strains SJL and BALB/c expressed cross-reacting idiotypes, whereas anti-nuclease (99-149)$_N$ antibodies from the same strains did not. This result was obtained using anti-idiotypic antisera prepared against either SJL or BALB/c antibodies. (*b*) Anti-nuclease (99-149)$_N$ from CB.20 and SJL shared a cross-reacting idiotype, whereas the anti-nuclease (1-99)$_N$ antibodies did not. Again, an identical result was obtained using the anti-idiotypic antisera prepared against SJL or B10.A(2R) antibodies. (*c*) Unlike BALB/c, strain BAB.14 expressed an anti-nuclease (99-149)$_N$ idiotype that cross-reacted with anti-nuclease (99-149)$_N$ from CB.20 and SJL. On the basis of these results, five distinct patterns of reactivity of antibody populations with the anti-idiotypic antisera could be distinguished. The idiotypes thus identified were termed NASE idiotypes and were identified by sequential letters A through E (Table II).

Discussion

The results presented in this communication confirm and extend our previous finding on the strain distribution of anti-nuclease idiotypes. By fractionation of antibodies to individual antigenic regions of the nuclease molecules, we have

TABLE II
Strain Distribution of NASE Idiotype Markers

Antigenic determinant	Idiotype markers	Positive strains
Nuclease $(1\text{-}99)_N$	NASE-A	A/J, BALB/c, BAB.14
	NASE-B	SJL, BALB/c
	NASE-C	CB.20, B10
Nuclease $(99\text{-}149)_N$	NASE-D	A/J, BALB/c, BAB.14
	NASE-E	SJL, CB.20, B10, BAB.14

Various anti-nuclease idiotypes, designated NASE markers, are listed along with their strain distribution and the antigenic region to which they are directed.

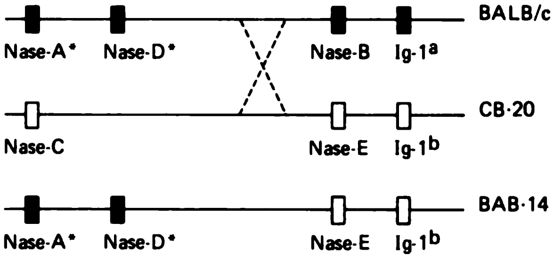

FIG. 2. Proposed map of genes determining anti-nuclease idiotypes. Map positions of genes for various NASE idiotype markers were assigned to account for results obtained on idiotype determinations of anti-nuclease antisera from strains BALB/c, CB.20, and the recombinant strain BAB.14. Distances with respect to the IgCH allotype locus as well as the relative positions of the NASE-A and NASE-D markers are arbitrary. The recombinational event that resulted in the BAB.14 strain is shown as involving the strain CB.20, as this strain was the source of antibodies used for the idiotype determinations. * indicates that position of these markers relative to each other is arbitrary.

been able to identify five distinct anti-nuclease idiotypes. These idiotypes can be distinguished by the antigenic region to which they are directed (either nuclease [1-99] or nuclease [99-149]) and by their strain distribution. This number of idiotypes represents the minimum number needed to account for the observed results, and it is likely an underestimate of the true number of anti-nuclease idiotypes present in immune sera. For each of five strains examined, a unique pattern of expression of these idiotypes has been identified.

For three of these strains, BALB/c, CB.20, and BAB.14, it is possible to map the genes that determine the different anti-nuclease idiotypes. To construct such a map, it is necessary that the genes determining these idiotypes all be linked to the C_H allotype locus. Since these three strains are congenic, with known genetic differences only at the C_H allotype locus and closely linked genes, the differences in their idiotype expression can be accounted for entirely on the basis of the linkage of idiotype genes to the C_H allotype locus. This linkage, however, does not imply that the structures recognized by the anti-idiotypic antisera are present exclusively on the heavy chain, nor that the gene linked to the C_H allotype locus is a structural rather than a regulatory gene.

Fig. 2 shows maps of idiotype genes for strains BALB/c, CB.20, and BAB.14

using the NASE idiotype markers defined in Table II. In these maps, the recombination event that occurred during the development of the BAB.14 strain is illustrated as involving the CB.20 strain, since the CB.20 strain was the source of antibodies of the B6 allotype used in the idiotype determination. A precise designation of the actual strain involved in this event cannot be given, since the time of occurrence of the recombination is unknown. These maps indicate that the crossover point between the BALB/c and B6 (CB.20) chromosomes occurred among the V_H genes rather than between V_H and C_H genes.

Two lines of evidence support this placement: (*a*) the BAB.14 strain has acquired the NASE-A and NASE-D markers from BALB/c, but not the NASE-B marker; (*b*) the BAB.14 strain expressed the NASE-E marker, which indicates that V_H region genes from B6 were acquired along with the $IgCH^b$ allotype locus. Since the BAB.14 strain has V_H genes from BALB/c and B6, the recombinational event must have occurred between different V_H region genes. At present, the position of the NASE markers can be made only relative to the crossover point and the C_H allotype locus. The map distances between the genes, as well as the ordering of NASE-A and NASE-D as shown is arbitrary. Nevertheless, such mapping is consistent with a linear array of V_H region genes.

These data also indicate that genes determining antibodies directed against the same antigenic regions of the nuclease molecule may have different map positions in different strains. Several examples are evident from our maps: (*a*) the genes determining anti-nuclease $(99\text{-}149)_N$ antibodies in strain CB.20 (NASE-E idiotype) are close to the allotype locus, whereas genes determining anti-nuclease $(99\text{-}149)_N$ antibodies in BALB/c (NASE-D idiotype) are distant, being localized to the other side of crossover point; (*b*) genes determining anti-nuclease $(1\text{-}99)_N$ in BALB/c (NASE-B idiotype) are close to the allotype locus, whereas anti-nuclease $(1\text{-}99)_N$ idiotype in strain CB.20 (NASE-C) and strain BALB/c (NASE-A) are both more distant, distal to the postulated crossover point; (*c*) in the BAB.14 strain, genes for idiotypically distinct anti-nuclease $(99\text{-}149)_N$ idiotypes occur at different positions, on either side of the crossover point. These results are similar to those obtained by Berek et al. (16) for mapping anti-streptococcal idiotypes, and they support the notion that genes for antibodies to the same antigenic determinants may map at nonhomologous positions in different strains. This suggests that different anti-nuclease idiotypes are not necessarily allelic.

In a previous study (3), two interesting features of the strain distribution of anti-nuclease idiotypes were noted: first, strain BALB/c ($IgCH^a$) shared idiotypes with strain A/J ($IgCH^e$) and SJL ($IgCH^b$); and second, strain B10 ($IgCH^b$) did not share idiotypes with strain SJL ($IgCH^b$). From these data we speculated on possible mechanisms of gene evolution that could have led to this distribution. It is clear from the studies presented here that the situation is more complex. Thus, whereas BALB/c mice have anti-nuclease $(1\text{-}99)_N$ idiotypes in common with both A/J and SJL, the anti-nuclease $(99\text{-}149)_N$ idiotypes are shared only with strain A/J. Similarly, although strains SJL and CB.20 do not share idiotypes for anti-nuclease $(1\text{-}99)_N$ antibodies, they do share an idiotype for anti-nuclease $(99\text{-}149)_N$. These results suggest that genes coding for different antibodies to the same antigen may have evolved independently. For the B10 and SJL strains, two models to account for such independent evolutionary development can be suggested. In the first model, one can postulate that both strains derived from a common ancestor. As the strains evolved, however, there was divergence of only some of the V_H region genes that had been inherited in common in such a manner that genes for the anti-nuclease (1-99)$_N$ idiotypes in the two strains no longer coded for cross-reacting idiotypes. This sequence of gene evolution would be consistent with allelism of genes determining antibodies to the same antigenic region if the genes for anti-nuclease (1-99)$_N$ idiotypes in both strains derived from the same ancestral genes. It is equally plausible, however, that the genes for the two idiotypes evolved from different ancestral genes. Alternatively, in a second model, one can consider both strains to have evolved from different ancestors with different V-gene repertoires. One can then propose that by convergent evolution, genes coding for cross-reactive anti-nuclease (99-149)$_N$ idiotypes emerged in both strains, although not necessarily at the same chromosomal location. At the moment, the constraints placed upon this evolutionary development by C_H allotype and other genes, e.g. immune response genes or genes coding for light chains, are unknown. It is hoped that analysis of more idiotypic markers will give further insight into this problem.

Summary

Antibodies to staphylococcal nuclease have been fractionated into two populations on the basis of their ability to bind to the cyanogen bromide cleavage product of nuclease comprising the C-terminal portion of the molecule from the 99th to the 149th amino acid. The two populations of antibodies, anti-nuclease (1-99)$_N$ and anti-nuclease (99-149)$_N$, have been prepared from a variety of strains, and analyzed using anti-idiotypic antisera raised against whole anti-nuclease antibodies from strains A/J, SJL, BALB/c, and B10.A(2R). Anti-nuclease (1-99)$_N$ antibodies had the same pattern of reactivity with the anti-idiotypic antisera as did unfractionated antibodies, whereas a different pattern was found for anti-nuclease (99-149)$_N$ preparations. On the basis of these studies, five anti-nuclease idiotypes, designated NASE markers, have been identified and defined on the basis of their antigenic specificity and strain distribution. With these additional markers, it has been possible to provide more detailed maps of variable (V) region genes in the strains BALB/c, CB.20, and the recombinant BAB.14. A recombinational event between V region genes during the development of the BAB.14 strain is suggested by the positioning of these NASE markers.

The authors wish to thank Mr. Walter Lyles and Mr. Francis Jones for their excellent animal care, and Mrs. Marilyn Schoenfelder for her expert secretarial assistance.

Received for publication 30 January 1978.

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Brief Definitive Report

LOSS OF PROLIFERATIVE CAPACITY IN IMMUNOHEMOPOIETIC STEM CELLS CAUSED BY SERIAL TRANSPLANTATION RATHER THAN AGING*

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Normally functioning marrow stem cells have a high proliferative capacity, but can be serially transplanted only three to five times before that capacity is severely diminished (1-4). This loss may demonstrate the natural limit of stem cell proliferative capacity, but it may also result from damage caused by the unnatural procedure of transplantation. The proliferative capacity of nontransplanted stem cells is tested by repeatedly irradiating the animal containing them, and thus forcing the cells to regenerate repeatedly. Under these conditions, marrow stem cells start with a high capacity for regeneration which eventually diminishes with repeated irradiation, and the treated animals die (5). This may be caused by stem cell exhaustion or by other cumulative effects of the irradiation treatments.

The ambiguities caused by damage from the process of transplantation or from treatment to destroy stem cells are removed by comparing the proliferative capacities of stem cells from old and young individuals. This experiment also answers a question that is relevant to the health care of elderly individuals: is a significant amount of stem cell proliferative capacity used up during a lifetime of normal functioning?

Unfortunately, results in this area are conflicting. Some investigators find defects in stem cells from old individuals (6-8), whereas others report no difference in functional abilities of old and young stem cells (9-11). Recently, Albright and Makinodan (8) suggested that old stem cells cannot multiply as rapidly as can young stem cells immediately after transplantation, although they are able to multiply and differentiate into normally functional immunohemopoietic cells if given adequate time. This suggestion was supported by findings that the macroscopic spleen colonies produced by old stem cells contained fewer cells. It conflicted with the recent report by Ogden and Micklem (11) that young marrow cells identified by chromosome markers and mixed with equal numbers of old marrow cells showed no competitive advantage in irradiated recipients. Only two separate old vs. young pairs were used; the young having the advantage in one case, and the old in the other.

The system of mixing old and young marrow cells and determining which better repopulates irradiated recipients compares stem cell proliferative rates immediately after transplantation. If young stem cells are capable of multiplying more rapidly than old cells immediately after transplantation, as suggested by Albright and Makinodan (8), the young cells would have a competitive advantage in repopulating irradiated recipients. We therefore compared the repopulating ability in irradiated recipients of many different young-old pairs,

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* Supported by research grants AG-00594 and AG-00250 from the National Institute on Aging, and HL-16119 from the National Heart and Lung Institute.

and we also measured the rates of spleen colony growth, using stem cells from young and old donors. In both cases we also studied previously transplanted old and young marrow stem cell lines to directly compare the effects of aging and transplantation.

Materials and Methods

Mice. CBA/H-T6J mice carrying two translocated T6 chromosomes (2T6), (CBA/H-T6J × CBA/CaJ)F_1 mice carrying one translocated T6 chromosome (1T6), compatible CBA/CaJ (0T6) mice, (C57BL/6J × CBA/H-T6J)F_1 = B6CBAT6F_1 mice, and (C57BL/6J × CBA/CaJ)F_1 W^x/W^r = B6CBAF_1-W^x/W^r mice were bred and maintained at The Jackson Laboratory, which is fully accredited by The American Association for Accreditation of Laboratory Animal Care. Old mice ranged from 25 to 33 mo of age while young ones were 3-8 mo old. Care was taken in autopsy procedures to use only healthy old mice as previously described (10). The evening before the cells were injected, irradiated recipients received 750-850 R of total body X-irradiation as described previously (10).

Rates of Colony Growth. Macroscopic spleen colonies (12) were counted on unfixed spleens of lethally irradiated histocompatible mice 9 days after irradiation and intravenous infection of 1.0 × 10^5 marrow cells. Then spleen cells were suspended in 2.0 ml of Ks 74, a buffered salt solution (13), after dispersion with a glass tissue homogenizer. The homogenate was filtered through 100 mesh nylon cloth, removing small chunks of gelatinous stroma; aliquots were diluted and counted on a Coulter model ZBI electronic cell counter (Coulter Electronics Inc., Hialeah, Fla.).

Comparative Repopulating Ability. Marrow cells were removed by rinsing both femurs and tibias of each donor with chilled CMRL-1066 media (Grand Island Biological Co., Grand Island, N. Y.) or Ks 74 (13) driven into one end of the bone through a 23-gauge disposable needle. Chunks were broken up by dispersing them through a disposable plastic 1-ml syringe with no needle attached and with its outlet pressed gently against the bottom of a 4-ml sterile plastic tube. Marrow cells were stored on ice, counted as described above for spleen cells, and injected within 1-3 h after removal. Marrow cells from each 2T6 donor were mixed with equal numbers of cells from the same young 1T6 donor in each experiment, and they were injected intravenously into lethally irradiated 0T6 recipients. Amounts were adjusted to contain 3 × 10^6 marrow cells of each chromosomal type. This is a new procedure making it possible to compare the repopulating abilities of marrow cells from several 2T6 donors at once. For example, in four experiments, the 2T6 donors included not only a young and an old individual, but also two irradiated 0T6 recipients previously populated with a young and with an old 2T6 stem cell line, respectively. Cells from all four 2T6 donors were mixed with cells from the same young 1T6 donor. Thus, the percentage of 2T6 cells in each case gave the relative proliferative ability of marrow stem cells from each 2T6 donor immediately after transplantation by measuring how well they competed with the 1T6 cells.

Recipients of the mixtures of 2T6 and 1T6 marrow cells were used 3-16 mo after irradiation and marrow transplantation. Their spleens were removed under anesthesia, and the numbers of T6 chromosomes in mitotic spleen cells were determined after stimulation in vitro with phytohemagglutinin (PHA) by methods previously described in detail (10). Erythropoietic mitoses were stimulated by removing 25-35% of the recipient's blood; marrow cells were removed for study 3 days later. Cells in mitosis were fixed in metaphase for determination of numbers of T6 chromosomes using Ford's methods (14). Only those cells with all 40 chromosomes clearly distinguishable were scored.

Results

There were no differences between young and old donors in colony-forming unit (CFU) numbers (*A*), numbers of cells per spleen (*B*), growth potentials (A × B), or cells per CFU (B/A) in mice of the two genotypes tested (Table I) at the initial transplantation. With CBA mice at the third transplantation, the growth potential of colonies from old donors was 2.4 times that of the young; with B6CBAF_1 mice at the second and third transplantations, the growth potential of colonies from young donors was 1.5-1.6 times that of the old (Table I).

TABLE I

Macroscopic Colony Growth in Irradiated Recipient Spleens

Donor		Recipient splenic values (mean ± SE)			
Age	Transplant	(A) Number of colonies	(B) Cell number	A × B	B/A
CBA mice*					
Old	1	6.6 ± 0.4	13.2 ± 7.1	96 ± 58	1.8 ± 0.9
Young	1	7.0 ± 0.7	13.1 ± 3.8	99 ± 36	1.8 ± 0.4
Old	2	6.9 ± 0.3	5.4 ± 0.2	37 ± 3	0.8 ± 0.1
Young	2	6.2 ± 0.7	6.1 ± 2.1	41 ± 18	1.0 ± 0.2
Old	3	6.2 ± 1.0	6.5 ± 1.7	43 ± 15	1.0 ± 0.1
Young	3	4.0 ± 1.5	3.5 ± 1.6	18 ± 9	0.8 ± 0.1
B6CBAF$_1$ mice‡					
Old	1	8.1 ± 0.9	25.0 ± 5.9	224 ± 60	2.9 ± 0.5
Young	1	7.6 ± 0.5	27.8 ± 4.8	220 ± 54	3.6 ± 0.4
Old	2	6.4 ± 0.6	14.2 ± 1.6	88 ± 5	2.4 ± 0.5
Young	2	7.4 ± 0.6	19.0 ± 1.3	142 ± 17	2.6 ± 0.3
Old	3	4.7	10.0	47	2.1
Young	3	5.0	14.0	70	2.8

Each mean ± SE is for 3 or 4 donors with CBA mice or 5 donors with B6CBAF$_1$ mice in three experiments. Values for individual donors were the means of 5 or 6 irradiated recipients of 1.0 × 10^6 marrow cells each (i.v.) per donor. (A) stands for number of colonies, and (B) for cell number (× 10^6) per spleen given after subtracting values for identical irradiated recipients injected with suspending media containing no cells for CBA recipients, or containing 1.0 × 10^6 W^x/W^v cells for B6CBAF$_1$ recipients.

(A) and (B) are multiplied (A × B) to give the growth potential, and divided (B/A) to give the number of cells per colony.

* Marrow stem cell lines from CBA/HT6 donors were carried in lethally irradiated CBA/CaJ recipients for transplants 2 and 3, using 4–10 × 10^6 marrow cells per recipient and 4–12 mo between serial transplantations. Age in months given as mean ± SE (n): for old donors, 25.7 ± 0.5 (7); for young donors, 5.0 ± 0.6 (8).

‡ Marrow stem cell lines from B6CBAT6F$_1$ donors were carried in unirradiated B6CBAF$_1$ – W^x/W^v recipients for transplant 2 using 10 × 10^6 marrow cells per recipient with 11–13 mo between serial transplantations. The same number of cells with 4 mo was used for transplant 3. Age in months is given as mean ± SE (n): for old donors, 29.0 ± 0.8 (8); for young donors, 7.4 ± 0.3 (8).

Much larger differences were evident with increasing numbers of serial transplantations than with increasing age. The number of cells per spleen, growth potentials, and number of cells per colony dropped to 40–70% when stem cell lines serially transplanted once (transplant 2, Table I) were compared with those never transplanted before (transplant 1, Table I). Colony numbers stayed constant between transplant 1 and 2, but generally declined in transplant 3, causing declines in the number of cells per spleen and in growth potentials; the number of cells per colony remained constant between transplants 2 and 3 (Table I).

The percentage of 2T6 cells which populated irradiated recipients in competition with 1T6 cells diminished to a much greater extent in donors whose cells were previously transplanted once (transplant 2) than in old donors (Table II). When mitoses were stimulated by bleeding, there was no difference between cells from old and young 2T6 donors (transplant 1, Table II), but there was a two- to fourfold decline in the ability to compete with cells from the 1T6 donor in stem cell lines that had been transplanted once previously (Transpl. 2, Table II). When mitoses were stimulated by PHA in vitro, stem cell lines from old 2T6 donors showed a slight defect, producing about 70% of the 2T6 cells of young 2T6 donors. However, stem cell lines of both age groups serially transplanted once competed 4- to 10-fold less well than those not previously transplanted (Table II).

TABLE II

Percentage of 2T6 Cells in Lethally Irradiated Recipients of 2T6 and 1T6 Marrow

2T6 Donors		2T6 Mitoses after	
Age	Transplant	Bleeding	PHA
Old	1	55 ± 5 (23)	33 ± 4 (16)*
Young	1	58 ± 4 (21)	47 ± 4 (18)
Old	2	27 ± 8 (6)‡	9 ± 3 (5)‡
Young	2	13 ± 4 (5)‡	4 ± 1 (4)‡

Results are given as mean ± SE (number of recipients scored). A mean of 45 (range 30–64) mitoses was scored for each recipient. Old or young 2T6 (CBA/HT6J) marrow was mixed with equal amounts (3×10^6 cells each) of young 1T6 (CBA/HT6J × CBA/CaJ) marrow and transplanted into lethally irradiated young 0T6 (CBA/CaJ) recipients in transplant 1. In transplant 2, marrow from the 2T6 donors had been previously injected i.v. ($4–10 \times 10^6$ cells) in 0T6 recipients; these recipients were held for 4–8 mo before they were used as donors. Age in months is given as mean ± SE (n): for old 2T6 donors, 28.3 ± 0.6 (16); for young 2T6 donors, 5.4 ± 0.3 (15); for young 1T6 competitors, 5.4 ± 0.3 (7).

* Significantly lower $0.01 < P < 0.05$ by Student-Neuman-Keuls multiple range test.

‡ Significantly lower $P < 0.01$ by Student-Neuman-Keuls multiple range test.

Discussion

We found no loss in growth potential of colony-forming cells from old mice (Table I), directly contradicting Albright and Makinodan (8) who found a 2.5- to 5-fold loss using mice of three different strains. There are three possible reasons for this contradiction. First, we calculated growth potential by a different method, multiplying total colony numbers per spleen by the total number of cells in the spleen which resulted from the injected marrow cells (above background). We used this procedure because essentially all proliferating cells in such a spleen are part of the colony and are of donor origin (8). Albright and Makinodan (8) calculated growth potential by multiplying the total colony number per spleen by the number of cells in the largest colony. This could give falsely high growth potentials for young marrow cells if they produced colonies with greater variability in size than were produced by old marrow cells. Second, the old donors we used may have been more free of factors suppressing colony growth because of differences either in general animal health or in autopsy procedures. Third, we used different strains of mice which may differ in the effects of age on stem cells. Our results are consistent with the finding of Lajtha and Schofield (15) that the growth rates of colonies from young or old donors do not differ significantly during the first 11 days after grafting, even though we measured total numbers of cells and they measured total numbers of colony-forming cells in the recipient spleens.

Our findings that old and young hemopoietic stem cells have equally potent proliferative capacities immediately after transplantation confirm and extend findings reported by Ogden and Micklem (11). These investigators studied recipients from only 2 pairs of old and young donors (11); in contrast, we compared 15 pairs of old vs. young donors from the CBA/H-T6 and the $B6CBAT6F_1$ genotypes in Table I, and 15 pairs from the CBA/H-T6 strain in Table II. We maximized the number of old donors and young controls because old individuals are highly variable (10), and we wanted to avoid being misled by a rare old individual that gave extreme results.

The evidence that stem cells from old mice were slightly defective in producing PHA-responsive cells (Table II) confirms results previously reported (10). This defect apparently resulted from stem cell residence in the old animal rather than being intrinsic to the stem cells, since it was not present in old stem cells in transplant 2 (Table II).

Although old and young stem cell lines gave similar results, the techniques we used were sensitive enough to detect large defects in stem cell lines after a single serial transplantation, even though we transplanted high numbers of marrow cells (4–10 × 10^6) and waited a lengthy interval (4–12 mo) before the second transplantation. Numbers of spleen cells but not numbers of colonies declined 1.5- to 2.5-fold after one serial transplantation (Table I). The ability to populate irradiated recipients declined even more strikingly, 2- to 4-fold in cells stimulated by bleeding, and 4- to 10-fold in cells stimulated by PHA (Table II). When such high marrow cell numbers and long transplantation intervals are used, other techniques do not detect striking changes until after 2–4 serial transplantations (2–4, 9, 11).

These results suggest that even one transplant is a much more severe stress for marrow stem cells than is normal functioning during a lifetime. The stress may result from damage by the transplantation process, dilution of a nonproliferating reserve of the earliest stem cells, exhaustion of proliferative capacity, or some combination of these. Little or none of the proliferative capacity in immunohemopoietic stem cell lines of mouse marrow appears to be used up by normal functioning throughout the adult lifespan.

Summary

Marrow stem cell lines from old donors and those from young controls gave equally rapid rates of colony growth on spleens of irradiated mice. Old and young stem cell lines competed equally well with chromosomally marked marrow stem cells from a young donor in producing cell types that are stimulated by bleeding; old cells competed 70% as well as young in producing cell types stimulated by phytohemagglutinin (PHA) in vitro. After a single serial transplantation, the rates of colony growth declined 1.5- to 2.5-fold, and the ability to compete declined 2- to 4-fold for bleeding-stimulated and 4- to 10-fold for PHA-stimulated cells. Thus, immediate stem cell proliferative capacities decline much more after one serial transplantation than after a lifetime of normal function.

Received for publication 6 February 1978.

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CONTENTS THE JOURNAL OF EXPERIMENTAL MEDICINE

Volume 147 No. 5 May 1, 1978

1299 **Giorgio Trinchieri, Daniela Santoli, Robert R. Dee, and Barbara B. Knowles.** Anti-viral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Identification of the anti-viral activity as interferon and characterization of the human effector lymphocyte subpopulation

1314 **Giorgio Trinchieri and Daniela Santoli.** Anti-viral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Enhancement of human natural killer cell activity by interferon and antagonistic inhibition of susceptibility of target cells to lysis

1334 **Joyce M. Zarling and Fritz H. Bach.** Sensitization of lymphocytes against pooled allogeneic cells. I. Generation of cytotoxicity against autologous human lymphoblastoid cell lines

1341 **U. Krawinkel, M. Cramer, I. Melchers, T. Imanishi-Kari, and K. Rajewsky.** Isolated hapten-binding receptors of sensitized lymphocytes. III. Evidence for idiotypic restriction of T-cell receptors

1348 **Lilly Y. W. Bourguignon, Robert Hyman, Ian Trowbridge, and S. J. Singer.** Localization of T25 glycoprotein in wild-type and Thy 1^- mutant cells by immunofluorescence and immunoelectron microscopy

1355 **P. R. Narayanan and G. Sundharadas.** Differential effects of polyadenylic: polyuridylic acid and lipopolysaccharide on the generation of cytotoxic T lymphocytes

1363 **Thomas G. Gipson, Masahiko Imamura, McGregor A. Conliffe, and W. John Martin.** Lung tumor-associated derepressed alloantigen coded for by the *K* region of the *H-2* major histocompatibility complex

1374 **I. Zan-Bar, E. S. Vitetta, F. Assisi, and S. Strober.** The relationship between surface immunoglobulin isotype and immune function of murine B lymphocytes. III. Expression of a single predominant isotype on primed and unprimed B cells

1395 **Peter J. Wettstein, Donald W. Bailey, Larry E. Mobraaten, Jan Klein, and Jeffrey A. Frelinger.** T-lymphocyte response to *H-2* mutants. I. Proliferation is dependent on Ly 1^+2^+ cells

1405 **Carlo E. Grossi, Susan R. Webb, Antonio Zicca, Peter M. Lydyard, Lorenzo Moretta, M. Cristina Mingari, and Max D. Cooper.** Morphological and histochemical analyses of two human T-cell subpopulations bearing receptors for IgM or IgG

1418 **Arthur K. Kimura and Hans Wigzell.** Cell surface glycoproteins of murine cytotoxic T lymphocytes. I. T 145, a new cell surface glycoprotein selectively expressed on Ly 1^-2^+ cytotoxic T lymphocytes

1435 **U. Botzenhardt, J. Klein, and M. Ziff.** Primary in vitro cell-mediated lympholysis reaction of NZB mice against unmodified targets syngeneic at the major histocompatibility complex

1449 **Paul S. Russell, Catharine M. Chase, Robert B. Colvin, and Janet M. D. Plate.** Kidney transplants in mice. An analysis of the immune status of mice bearing long-term, H-2 incompatible transplants

1469 **Paul S. Russell, Catharine M. Chase, Robert B. Colvin, and Janet M. D. Plate.** Induced immune destruction of long-surviving, H-2 incompatible kidney transplants in mice

1487 **E. S. Raveché, A. D. Steinberg, L. W. Klassen, and J. H. Tjio.** Genetic studies in NZB mice. I. Spontaneous autoantibody production

1503 **Phyllis Bodel.** Spontaneous pyrogen production by mouse histiocytic and myelomonocytic tumor cell lines in vitro

1517 **David S. Pisetsky and David H. Sachs.** Genetic control of the immune response to staphylococcal nuclease. VIII. Mapping of genes for antibodies to different antigenic regions of nuclease

BRIEF DEFINITIVE REPORT

1526 **David E. Harrison, Clinton M. Astle, and Joan A. Delaittre.** Loss of proliferative capacity in immunohemopoietic stem cells caused by serial transplantation rather than *aging*

R 11
J57

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VOLUME 147, NO. 6 JUNE 1, 1978

JEMEAV 147(6) 1535-1882 (1978) • ISSN 0022-1007

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IgG_{2a}-PRODUCING VARIANTS OF AN IgG_{2b}-PRODUCING MOUSE MYELOMA CELL LINE*

BY TOVA FRANCUS,‡ B. DHARMGRONGARTAMA, RICHARD CAMPBELL, MATTHEW D. SCHARFF, AND BARBARA K. BIRSHTEIN§

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The expression of immunoglobulin genes is usually unstable in mouse myeloma cells (1, 2). Some cell lines spontaneously lose the ability to produce immunoglobulin heavy chain at a rate of 1 × 10^{-3}/cell/generation (3). One cell line, P3 (MOPC 21), has yielded spontaneous variants which synthesize altered heavy chains. Adetugbo and co-workers have examined 7,000 P3 clones and have identified four such variants, three of which have deletions, and one which has a point mutation (4).

After mutagenesis of the MPC 11 mouse myeloma cell line, as many as 2–6% of the surviving cells are variants in heavy chain production, of which approximately two-thirds have ceased synthesis of heavy chains while continuing light chain production (5). The remaining third of these MPC 11 variants synthesize altered heavy chains and are divided about evenly between two major types: (*a*) those synthesizing heavy chains shorter than the parent (5); and (*b*) those synthesizing heavy chains which lack the γ2b serologic determinants of the parental heavy chain but which express serological determinants of the γ2a subclass (6).

Identification of the original primary variants which produced a γ2a heavy chain was based on serology, assembly patterns, and peptide maps (6). Recently, chemical characterization and partial amino acid sequence determination of one γ2a variant protein has shown that the Fc portion of its heavy chain is distinct from that of MPC 11, the parental IgG_{2b} immunoglobulin, and is identical to that of MOPC 173 (7), an $IgG_{2a}(\kappa)$ mouse myeloma protein of known sequence (8). These γ2a variant heavy chains are identical in size to the parent with the exception of one, which is larger. The latter variant gives rise to secondary variants producing γ2a heavy chains of normal size (9). Additional secondary variants producing γ2a heavy chains of normal size have been derived from variants synthesizing short heavy chains (9, this paper).

Previous instances of the production of a new subclass in immunoglobulin-producing cells have been reported. For example, Hausman and Bosma have described two cases in which myeloma tumors ceased production of the original

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* Supported by grants NIH AI 13509, NIH AI 10702 from the National Institutes of Health.

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heavy or light chain and began synthesis of a new chain differing in subclass or type, and binding of idiotype (10). However, both these changes appeared during adaptation to continuous in vitro culture by alternate passage between culture and animal, and the origin of the variant cell was thus in doubt. In addition, Kunkel et al., Natvig and Kunkel, and Werner and Steinberg have observed the presence in human serum of "hybrid" immunoglobulins which contain antigenic determinants of two heavy chain subclasses (11-13). A similar observation has been made in mice (14).

We do not know what genetic or biochemical mechanisms are responsible for the instability of immunoglobulin expression in mouse myeloma cells or if this instability is related to any of the normal events in the regulation of antibody production. Since the γ2a variants arise frequently, either directly from the parental cell line or as secondary variants from variants producing short heavy chains, and are expressing either part or all of a gene that was silent in the parent, a more detailed study of these variants should provide some insight into the structure of the immunoglobulin genes and the genetic control of immunoglobulin expression. In this paper, we examine the relationship of the γ2a variants to each other and to their parent.

Materials and Methods

Cell Lines and Tumors. All cell lines used in this study are summarized in Table I. The cell line, 45.6.2.4, was derived from the BALB/c mouse myeloma tumor, MPC 11, which synthesizes an IgG$_{2b}$(κ) immunoglobulin (15). Primary (6) and secondary (9) variants synthesizing IgG$_{2a}$(κ) immunoglobulins were obtained from MPC 11 by using a cloning technique (3). Additional variants synthesizing IgG$_{2a}$(κ) have been isolated during the course of these studies. ICR 4.68.66 and ICR 4.68.110 are secondary variants derived from the short heavy chain producing variant, ICR 4.68. U2 arose spontaneously. The parental cell line and all variants retained tumorigenicity, both as solid tumors and ascites. Cell lines were grown in suspension culture in Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, N.Y.), supplemented with 20% heat inactivated horse serum (Grand Island Biological Co., or Flow Laboratories, Inc., Rockville, Md.), nonessential amino acids, L-glutamine (3.33 mM), penicillin (10,000 U/liter), and streptomycin sulfate (50 mg/liter), all purchased from Grand Island Biological Co. MOPC 173, a gift from Dr. Melvin J. Bosma, Institute for Cancer Research, Philadelphia, Pa. and LPC 1, a gift of Dr. Michael Potter, NIH, Bethesda, Md., both IgG$_{2a}$ (κ), were maintained in BALB/c mice.

Preparation of Antisera. Rabbits were immunized by numerous intradermal injections in the flanks and footpads with a total of 100-250 μg protein emulsified by sonication in 1 ml of 50% complete Freud's adjuvant (Difco Laboratories, Detroit, Mich.). After 3 wk, rabbits were boosted with an intravenous injection (1 ml) of 100 μg protein coated onto 300 μg alumina particles. Beginning 1 wk after boosting, rabbits were bled twice a week until the antibody titer fell. The antisera were prepared against immunoglobulin from MPC 11 (IgG$_{2b}$), LPC 1 (IgG$_{2a}$), and ICR 9.9.2.1 (secondary variant, IgG$_{2a}$), and the Fc fragments of immunoglobulin from MPC 11 and LPC 1. Commercial antisera directed against the different classes and subclasses of mouse immunoglobulins were obtained from Meloy Laboratories, Inc., Springfield, Va. Their specificity was confirmed by using a panel of myeloma proteins.

Purification of Immunoglobulin. Cells were injected intraperitoneally into pristane (2,6,10,14-tetramethyl-pentadecane, Aldrich Chemical Co., Milwaukee, Wis.) primed BALB/c mice (16). The ascites from the tumor-bearing mice were collected, centrifuged to remove cells and debris, treated at 56°C for 30 min, and stored frozen. The presence of paraprotein was verified by cellulose acetate microzone electrophoresis (Beckman Microzone Electrophoresis System, Bulletin 7086, Beckman Instruments, Inc., Fullerton, Calif.).

The myeloma protein was precipitated with ammonium sulfate as described (17). Further purification was carried out by using ion exchange chromatography on a column of DEAE-cellulose (DE-52, Whatman, Inc., Clifton, N.J.). The buffers used for the linear gradient were a modification of Potter's system (17). The starting buffer was 0.005 M Tris-phosphate pH 8.6, and the final buffer was a 4:1 mixture of 0.005 M Tris-phosphate, pH 8.6, and 0.5 M Tris-phosphate, pH 5.1. The purified protein was dialyzed against distilled water and freeze-dried. The purity of the proteins was verified by microzone electrophoresis, immunoelectrophoresis, and polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE)[1] (18).

Radioimmunoassay

PREPARATION OF IMMUNOADSORBENT COLUMN. Sera from normal mice, mice bearing MOPC 141 tumor, and mice bearing MOPC 195 tumor (both IgG_{2b}, κ) were pooled and mixed with an equal volume of phosphate-buffered saline (0.15 M NaCl, 0.02 M sodium phosphate, pH 7). The serum pool was brought to 40% saturation by the addition of saturated ammonium sulfate and stirred at 4°C for 30 min. The precipitated proteins were dialyzed against 0.25 mM phosphoric acid-40 mM Tris and applied to a column of DEAE cellulose (DE-52, Whatman), equilibrated in the same buffer. The IgG fraction was eluted with 12.5 mM phosphoric acid-250 mM Tris and coupled to Sepharose 4B (19). The resulting immunoadsorbent contained 3.72 mg of IgG/ml Sepharose.

PREPARATION OF ANTI-IDIOTYPIC ANTISERA. Rabbit antisera against immunoglobulin from MPC 11 (R132) or ICR 9.9.2.1 (BB 24) were brought to 0.5 M in NaCl and chromatographed in this solution on the immunoadsorbant column to which normal IgG and IgG_{2b}, κ-myeloma proteins had been attached. The eluted (unbound) fractions were tested by Ouchterlony analysis against the immunizing antigen and normal mouse serum, MOPC 195, MOPC 141, and MOPC 173. Those fractions from R132 that were reactive only against the immunizing antigen MPC 11, and those fractions from BB 24 which were reactive only against the immunizing antigen ICR 9.9.2.1, were pooled, and concentrated, when necessary, by dialysis against Sephadex G-200 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N.J.).

PREPARATION OF RADIOLABELED MPC 11 IMMUNOGLOBULIN. MPC 11 cells (10^6/ml) were grown for 24 h in Spinner medium (20) containing 1/10 the normal concentration of methionine, 10% heat inactivated horse serum, and 100 μCi of ^{35}S-methionine/ml (New England Nuclear, Boston, Mass.). 200 μg/ml of unlabeled methionine were added to the supernate to replace nonspecifically absorbed [^{35}S]methionine. The supernate was then dialyzed against phosphate-buffered saline. We used the radiolabeled MPC 11 preparation without further purification since we found that it behaved identically to chromatographically purified radiolabeled protein in the radioimmunoassay.

RADIOIMMUNOASSAY. The solid phase radioimmunoassay of Bosma et al. (21) was followed except that bound radioactive proteins were released from the polystyrene tubes by sonication for 10 min in 1 ml of 1% SDS and transferred for counting to scintillation vials containing 10 ml Aquasol (New England Nuclear).

Electrophoresis in Agarose. The Johansson method (22), as modified by Dr. Chester Alper (personal communication) was used. The electrophoresis buffer (pH 8.6) was composed of 35.05 g sodium barbital and 5.5 g barbital in 4 liters. The fixing, staining, and destaining solutions were methanol:water:acetic acid (5:5:1, vol/vol/vol) with 0.02% Coomassie Brilliant Blue R-250 (Consolidated Laboratories Inc., Chicago Heights, Ill.) added to the staining solution.

Assembly of Immunoglobulin. Logarithmically growing cells (3 × 10^6) were washed twice in Spinner medium minus valine, threonine, leucine, and containing 2% heat-inactivated horse serum. The cells were preincubated in 1 ml medium for 5 min in a 37°C water bath. 5 μCi each of [^{14}C]L-valine, -threonine, and -leucine were added (New England Nuclear) and the cells were incubated further for 15 min. The incorporation of radioisotopes was stopped by immediate cooling of the cells in an ice bath and the addition of 1 ml cold medium (4°C). The cells were collected and lysed as previously described (5). Radiolabeled immunoglobulins were immunologically precipitated by an indirect technique (5) and were analyzed by 5% SDS-PAGE. Gels were dried and subjected to radioautography on Kodak XR-5 film (Eastman Kodak Co., Rochester, N.Y.). Radioautographs were scanned (log mode) with a reflectance fluorescence transmission (RFT) scanning microdensitometer equipped with an automatic General Computing Integrator (Transidyne General Corp., Ann Arbor, Mich.).

Preparation of Radiolabeled Secreted Immunoglobulin. Logarithmically growing cells were washed twice in Spinner medium containing 1/40 the normal amount of valine, threonine, and leucine, and supplemented with 10% heat inactivated horse serum. The cells were resuspended in the same medium at a final concentration of 5 × 10^5 cells/ml, and 5 ml were placed in a Petri dish. Radiolabeled L-valine, -threonine, and -leucine were added at 15 μCi ^{14}C each, or at 25 μCi ^{3}H each. The dishes were incubated at 37°C in 5% CO_2 for 48 h, at which time the cells were removed by centrifugation, the immunoglobulin was precipitated from the supernate by the sandwich technique, and heavy and light chains were prepared by using SDS-PAGE (5).

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[1] *Abbreviation used in this paper*: SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

A large quantity of radiolabeled ICR 9.9.2.1 immunoglobulin was prepared from 40 ml of cell suspension in a spinner culture, incubated with 250 μCi each of [^{3}H]L-valine, -threonine, and -leucine. The supernate was dialyzed against phosphate-buffered saline to remove labeled amino acids after which the protein was precipitated with 50% saturated ammonium sulfate. The precipitate was dissolved in phosphate-buffered saline, dialyzed against distilled water, and freeze-dried. The radioactivity in the precipitate was found exclusively in immunoglobulin (15). The protein was subjected to complete reduction and alkylation (23), and radiolabeled heavy and light chains were separated by gel filtration on a column of Sephadex G-100 (2.2 × 180 cm), equilibrated with 4.5 M urea, 1 M propionic acid (24). Elution was monitored by radioactivity. The pooled peaks were freed of salts by passage through a column of Sephadex G-25, coarse (3 × 60 cm), equilibrated with 0.05 M formic acid.

Papain Digestion. Radiolabeled Fab and Fc of ICR 11.8 and M224 were isolated after papain digestion as described by Guyer et al. (25).

Ion Exchange Chromatography of Peptides. A mixture of [^{3}H] and [^{14}C]labeled proteins was subjected to digestion by sequential additions of trypsin and chymotrypsin. The freeze-dried enzymatic digest (5) was dissolved in 1.5 ml of 0.3 M pyridine-HCl, pH 1.7, and the pH was adjusted to <pH 2 with glacial acetic acid. The peptides were applied to a heated (56°C), water-jacketed column (0.2 × 23 cm), packed with a Dowex-50 sulfonated polystyrene resin (SPHERIX, type XX907, Phoenix Precision Instrument Co., Philadelphia, Pa.) (15), which was equilibrated with 0.05 M pyridine-acetic acid, pH 3.13. The peptides were eluted with a gradient generated in a Varigrad (VirTis Co., Inc., Gardiner, N.Y.), by using 110 ml each of the following pyridine-acetic acid buffers: (*a*) 0.05 M, pH 3.13; (*b*) 0.10 M, pH 3.54; (*c*) 0.20 M, pH 4.02; (*d*) 0.5 M, pH 4.5; and (*e*) 2.0 M, pH 5.0 (26). 200 fractions of 2.5 ml each were collected into glass scintillation vials, the pH was recorded, the buffer was evaporated in an oven, and 0.5 ml H_2O and 10 ml Aquasol were added to each vial. The samples were counted in a Beckman scintillation counter (LS-230 or LS-233, Beckman Instruments, Inc., Fullerton, Calif.), with the isosets adjusted so that the spill from the ^{3}H to the ^{14}C channel was <0.001%, and the spill from the ^{14}C to the ^{3}H channel was 7 ± 1%. The data were corrected for background and spill, and plotted as cpm or percent of total counts vs. fraction number, by using a computer program developed for us by Mr. David Medford.

Results

Ouchterlony Analysis of Parent and Variant Immunoglobulins. Cytoplasms and secretions from cultured cells, and immunoglobulins purified from sera or ascites of tumor bearing mice were tested by double diffusion in agarose (5). All of the variants listed in Table I were γ2a positive and γ2b negative when tested with subclass specific (Meloy Laboratories Inc., Springfield, Va.) antisera. These γ2a immunoglobulins showed "complete identity" with each other using a variety of antisera produced against MPC 11 (IgG_{2b}, κ), LPC 1 (IgG_{2a}, κ), the γ2a variant protein—ICR 9.9.2.1, and the Fc fragments of MPC 11 and LPC 1. All the variants shared the same antigenic determinants with the parent and lacked some antigenic determinants which were present on MPC 11 (Fig. 1a). When tested with an antisera raised against the Fc fragment of LPC 1 (IgG_{2a}, κ), the variants shared the same determinants, some of which were absent from the parent (Fig. 1b). This analysis showed that at least part of a different constant region gene is expressed in these variants and distinguished the γ2a variant proteins as a group from the parent γ2b protein. Since many of the

TABLE I

The γ2a Producing Cell Lines Derived from MPC 11

Parent	Mutagen	*Primary variant	*Secondary variant	Heavy chain		
				Size	Serotype	Secretion
45.6.2.4				55,000	γ2b	+
45.6.2.4	–	U2		55,000	γ2a	+
[a]45.6.3.1	Melphalan	‡M224		55,000	γ2a	+
[b]45.6.3.1	Melphalan	M319.2		55,000	γ2a	+
[c]45.6.2.4	ICR 191	‡ICR 11.8		55,000	γ2a	+
[d]45.6.2.4	ICR 191	ICR 16		55,000	γ2a	+
[e]45.6.2.4	ICR 191	ICR 9		75,000	γ2a	–
		§ ICR 9.9		75,000	γ2a	–
			→ ICR 9.9.2.1	55,000	γ2a	+
			→ ICR 9.9.1.6.7	55,000	γ2a	+
45.6	ICR 191	ICR 4.68		50,000	–	–
			→ ICR 4.68.66	55,000	γ2a	+
			→ ICR 4.68.110	55,000	γ2a	+
[f]45.6.3.2	ICR 191	ICR 11.19		50,000	–	–
			→ ICR 11.19.2	55,000	γ2a	+
			→ [g]ICR 11.19.3	55,000	γ2a	+

* In the text, we use an abbreviated form of the cell lines starting with the letter M, or ICR.

‡ M and ICR indicate the variant was obtained after mutagenesis with Melphalan or ICR 191, respectively.

§ An arrow indicates that the subclone was obtained without further mutagenesis. a, b, c, d, and e were listed in Preud'homme et al. (6) as: 45.6.3.2.2 M224; 45.6.3.2.2 M319; 45.6.3.1 ICR 11; 45.6.3.1 ICR 16; 45.6 ICR 9; respectively. f was listed as 45.6.3.2 ICR 11 in Birshtein et al. (5). g was listed as 45.6.3.2 ICR 11.19.S3 in Koskimies and Birshtein (9).

antigenic determinants detected by these antisera are in the Fc region, the serology of the variable region was examined directly.

Idiotypic Analysis of Parent and Variant Immunoglobulins by Radioimmunoassay. Rabbit antiserum to MPC 11 (R 132) was absorbed on an immunoadsorbent column, as described in Materials and Methods. This anti-idiotypic antiserum bound to MPC 11, and the binding was not affected by normal mouse serum, two other IgG$_{2b}$(κ) proteins (MOPC 141 and MOPC 195) (Fig. 2), or the IgG$_{2a}$(κ) immunoglobulin, MOPC 173 (Fig. 3). In contrast, proteins purified from six γ2a-producing variants were identical to each other and to the parental MPC 11 protein in their ability to inhibit this binding to the radiolabeled MPC 11 protein (Fig. 3). A second rabbit antiserum (BB24) was raised against the γ2a variant protein, ICR 9.9.2.1, and was also made anti-idiotypic by appropriate absorption (Materials and Methods). In a similar radioimmunoassay, but

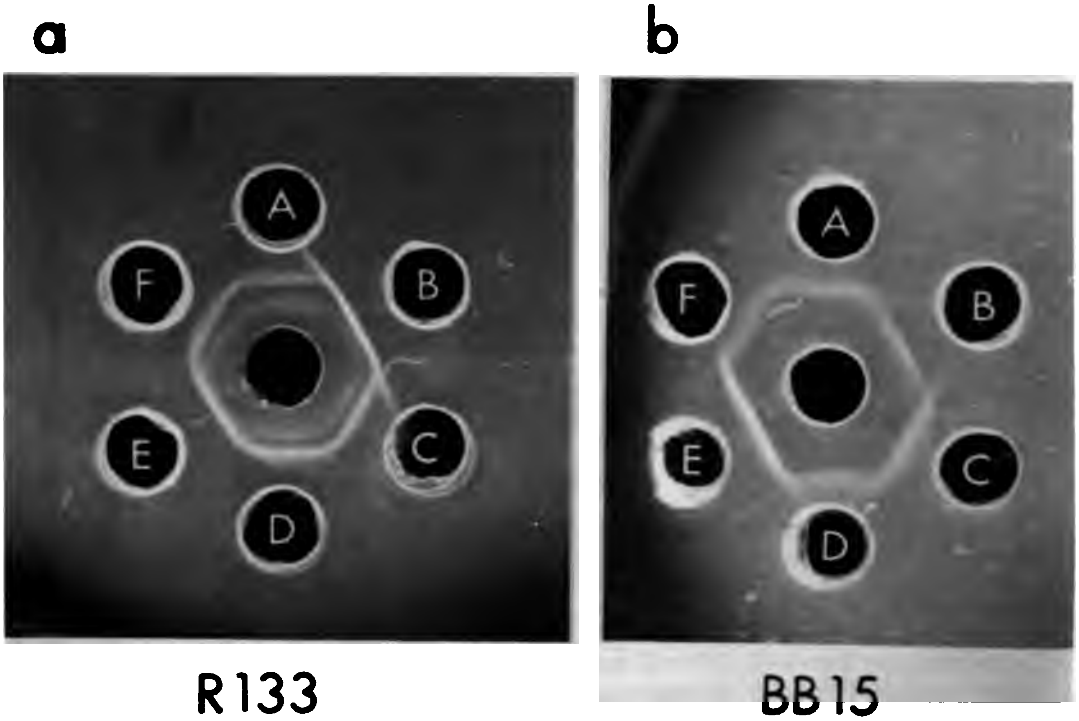

Fig. 1. Ouchterlony analysis of parent and IgG_{2a} variant proteins. A, ICR 9.9.2.1. B, MPC 11 (parent). C, ICR 11.8, D, M224. E, ICR 11.19.2. F, ICR 16. a, R 133 is anti-MPC 11. b, BB 15 is anti-LPC 1-Fc.

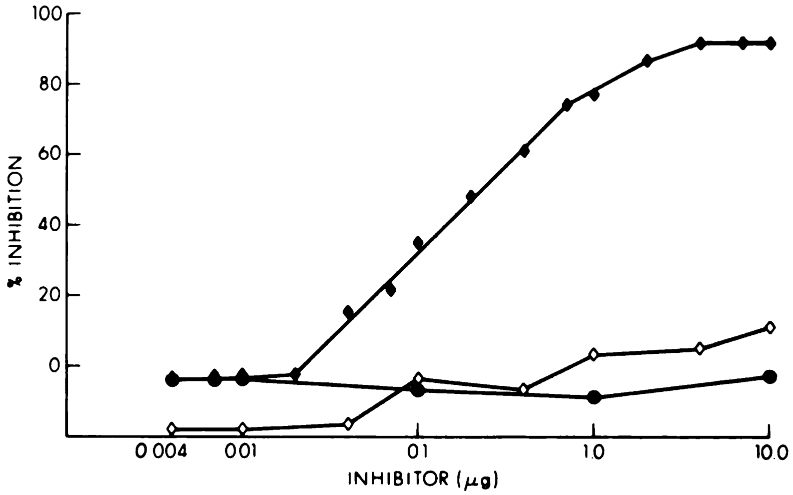

Fig. 2. Inhibition of the binding of [^{35}S]Met-MPC 11 to anti-idiotypic antisera (R 132) directed against MPC 11 by ◆ MPC 11, ◇ MOPC 141, ● MOPC 195.

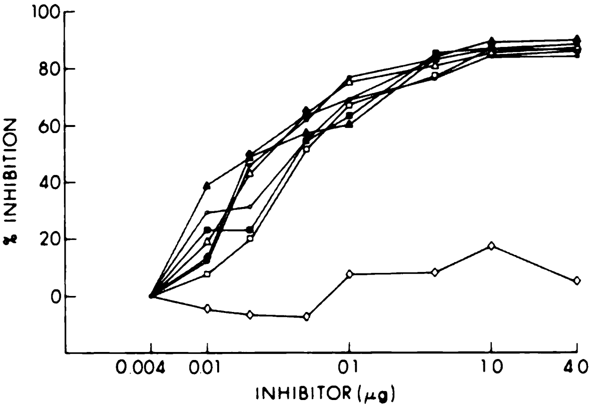

FIG. 3. Inhibition of binding of [^{35}S]Met-MPC 11 to anti-idiotypic antisera directed against MPC 11 by purified IgG$_{2a}$ variant proteins. ◆ MPC 11, ☐ M224, ■ ICR 16, ○ ICR 11.8, △ ICR 9.9.2.1, ▲ ICR 4.68.66, ● ICR 11.19.3, ◇ MOPC 173.

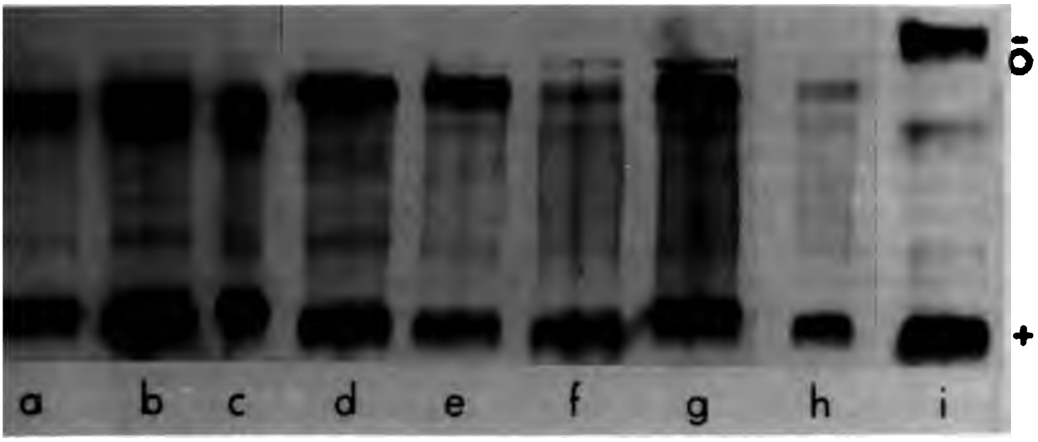

FIG. 4. Agarose gel electrophoresis of ascites. a, ICR 4.68.66. b, ICR 11.19.2. c, ICR 11.19.3. d, MPC 11. e, ICR 11.8. f, ICR 16. g, M224. h, ICR 9.9.2.1. i, MOPC 173. O-origin. The major protein band near the anode is albumin.

using a smaller set of proteins, MOPC 173 did not inhibit the binding of MPC 11 to the second antiserum while the parent, MPC 11, and two variants, ICR 9.9.2.1 and ICR 11.8, inhibited equally well (data not shown). These results showed that the γ2a variant proteins contained MPC 11 variable region antigenic determinant(s).

Electrophoretic Migrations of Variant Proteins in Agarose Gels. To try to discriminate between the variants, we examined ascites from the different γ2a producing variants, from MPC 11, and from MOPC 173 by electrophoresis on agarose plates. The γ2a paraproteins from the variants clearly fell into two groups (Fig. 4). The first group consisted of the secondary variants derived independently from two primary variants (ICR 4.68 and ICR 11.19) which produced heavy chains with a mol wt of 50,000 (Table I). These four proteins, three of which are shown in Fig. 4, panels a–c, were more negatively charged

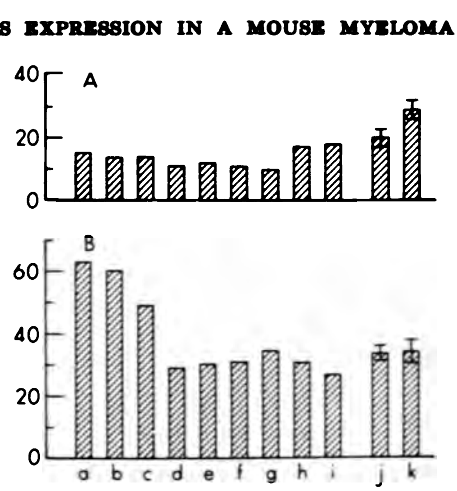

Fig. 8. Assembly patterns of $\gamma 2a$ producing variants compared by normalized HL (A), and normalized H_2L_2 (B). a, ICR 4.68.66. b, ICR 11.19.2. c, ICR 16. d, ICR 11.8. e, M224. f, M319.2. g, ICR 9.9.2.1. h, ICR 9.9.1.6.7. i, U2. j, LPC 1 ($\gamma 2a$ control). k, MPC 11 (parent $\gamma 2b$). Variants were compared by using integration of densitometric traces. Calculations were normalized to only those assembly components containing heavy chains because of varying amounts of light chains produced by different variants; e.g. normalized H_2L_2 = $H_2L_2/(H_2L_2 + H_2L + H_2 + HL)$; normalized HL = $HL/(H_2L_2 + H_2L + H_2 + HL)$.

the HL intermediate as their major pathway of assembly.

Fig. 8B shows the histogram of normalized H_2L_2 as a measure of the rate of assembly. $\gamma 2a$ producing variants are shown in panels a–i (ICR 4.68.66, panel a, and ICR 11.8, panel d) and the results of four determinations each of LPC 1 and MPC 11 are shown in panels j and k, respectively. Visually, the $\gamma 2a$ variants differ from each other much more than the error of the procedure. Statistically, with 95% confidence,[4] we can say that the $\gamma 2a$ variants fall into more than one group. Those variants having the fastest rate of assembly are ICR 4.68.66 (panel a), ICR 11.19.2 (panel b), and ICR 16 (panel c). Of these three, ICR 4.68.66 and ICR 11.19.2, both secondary $\gamma 2a$ variants derived from short heavy chain producing variants, were previously grouped together on the basis of charge and peptide maps; while ICR 16 has seemed to be the most distinctive of the $\gamma 2a$ producing variants because of its peptide profile (Fig. 5a) (6).

The remaining six variants (Fig. 8B, panels d–i) have a similar rate of assembly which does not differ significantly from either MPC 11 ($\gamma 2b$) or LPC 1

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[4] $\overline{H_2L_2} = H_2L_2/(H_2L_2 + H_2L + H_2 + HL)$ = fraction H_2L_2 is of total assembly components containing heavy chains; $\overline{HL} = HL/(H_2L_2 + H_2L + H_2 + HL)$ = fraction HL is of total assembly components containing heavy chains; $S_1{}^2{}_{\overline{H_2L_2}}$, $S_1{}^2{}_{\overline{HL}}$—the variance of $\overline{H_2L_2}$ and $\overline{HL}$, respectively, as calculated for the percent assembled H_2L_2 and HL from four independent labeling experiments of MPC 11. The subscript "1" indicates control. $S_2{}^2{}_{\overline{H_2L_2}}$, $S_2{}^2{}_{\overline{HL}}$—the variance of $\overline{H_2L_2}$ and $\overline{HL}$, respectively, in the nine variants studied for the percent assembled H_2L_2 and HL. Subscript "2" indicates variants. $F_{\overline{H_2L_2}} = S_2{}^2{}_{\overline{H_2L_2}}/S_1{}^2{}_{\overline{H_2L_2}} = 194.58/12.86 = 15.13$; $F_{\overline{HL}} = S_2{}^2{}_{\overline{HL}}/S_1{}^2{}_{\overline{HL}} = 8.6/0.7 = 12.28$; critical value for $F_{.05}$ (8, 3) = 8.84. Since both $F_{\overline{H_2L_2}}$ and $F_{\overline{HL}}$ are > 8.84, we can say at the 95%+ confidence level that the variants fall into more than one assembly group. That is, under the null hypothesis that the variants fall into one group and assuming the experimental errors are the same for the variants and the controls, the true variance of the control $\sigma_1{}^2{}_{\overline{H_2L_2}}$ will equal the true variance of the variants $\sigma_2{}^2{}_{\overline{H_2L_2}}$. Similarly, $\sigma_1{}^2{}_{\overline{HL}}$ will equal $\sigma_2{}^2{}_{\overline{HL}}$. The sample F values of 15.13 and 12.28 allow us to reject the null hypothesis that the variants fall into one group.

(γ2a). It is noteworthy that ICR 11.8, M224, and M319.2 (panels d, e and f, respectively), the three variants that seem indistinguishable by peptide maps, assemble similarly.

Discussion

We have isolated from the γ2b producing cell line, MPC 11, a dozen variants synthesizing immunoglobulins having a γ2a serotype. The expression of the γ2a structural gene was confirmed when we showed that the Fc region of one variant was identical to the Fc of the IgG_{2a} immunoglobulin MOPC 173, and different from that of the IgG_{2b} immunoglobulin, MPC 11 (7).[2] In this paper, we have shown, by a radioimmunoassay, that the idiotypes of the variants are identical to those of the parent. The simplest explanation for the generation of variants synthesizing heavy chains which have the parental idiotype and a constant region of a previously silent gene is a translocation of the sort suggested by Gally and Edelman (28). This hypothesis would predict that all the γ2a variants would be identical to each other. However, we showed that two γ2a variants (M224 and ICR 16) differed extensively in their peptide profiles (6). This finding suggested that other genetic mechanisms must be involved. Any genetic mechanism proposed must take into account the high incidence of variants and the multiple routes by which they arose. Primary γ2a producing variants generated by either ICR 191 or Melphalan occurred at an incidence of 0.6–2% (6) and secondary variants arose without further mutagenesis at the same frequency (9).

Our focus here was to define differences and similarities among the γ2a variants that might allow us to distinguish and group them and understand the genetic mechanisms of their origins. The major outcome of these studies has been the observation that these many γ2a variant proteins differ from each other. However, our examination of the variants by charge, peptide maps and assembly characteristics has enabled us to subdivide them. Three of the primary variants – M224, M319.2 and ICR 11.8 – are very similar by all our assays and, in fact, are almost indistinguishable by peptide maps. It should be emphasized that two of these variants arose after Melphalan mutagenesis and the other after ICR-191 treatment. This group might thus result from a commonly operative mechanism in an immunoglobulin producing cell. Translocation is one such mechanism, but only detailed structural studies will enable us to test this hypothesis.

A second group is comprised of the secondary variants which arose from ICR 4.68 and ICR 11.19, which are primary variants synthesizing short heavy chains of mol wt 50,000. These secondary variants – ICR 4.68.66, ICR 4.68.110, ICR 11.19.2 and ICR 11.19.3 – were similar in charge and in assembly pattern. Their peptide maps showed extensive similarities; however, they are not identical.

The rest of the variants do not fall into obvious groups. ICR 16 and ICR 9, both primary variants obtained after ICR 191 treatment, differ markedly from the other γ2a variants, including ICR 11.8, which arose after identical treatment. ICR 16's structure is probably the most different of the γ2a variants, as reflected by its peptide maps and its assembly pattern. ICR 9 is unique by virtue of its 75,000 mol wt heavy chain. The secondary variants derived from ICR 9 – ICR 9.9.2.1 and ICR 9.9.1.6.7 – could potentially comprise a group since their peptide maps show similarities; but their assembly patterns are rather different.

The spontaneously arising variant, U2, differs in peptide map and assembly pattern and also falls outside of our groupings. Our finding that several γ2a variants differ thus precludes strict translocation as a sole mechanism for their generation.

We considered the possibility that the variants represent the expression of different γ2a constant region genes which are ordinarily silent similar to the observation by Bosma and Bosma of the "wrong" allotype in a congenic mouse (29). However, our findings would seem to require more γ2a constant region genes that one might propose on the basis of nucleic acid hybridization data for light chain genes. In addition, Dr. Melvin Bosma and Ms. Carol DeWitt (unpublished data) showed that all our variants carried the BALB/c γ2a allotype.

It is possible that all our variants could arise by a common mechanism, such as a series of recombination events between γ2b and γ2a constant region genes and that preferred sites of recombination ("hot spots") could explain the high frequency of some types of variants. By this mechanism, one would expect the variant heavy chains to be hybrid molecules containing varying lengths of γ2b and γ2a constant region sequences. Such hybrid molecules have been reported (11-14). Thus far, we have not serologically detected any γ2b determinants in these variants; nor have we found any γ2b specific residues in our sequence studies of the Fc of one variant, ICR 9.9.2.1. Recently, however, we have identified such residues in another γ2a variant protein, ICR 11.19.3 (M. L. Greenberg, R. Campbell, and B. K. Birshtein, unpublished data). Whether our variants reflect the type of DNA sequence arrangements recently seen in genes coding for proteins as diverse as adenovirus, globin, and immunoglobulin (30, 31) remains, of course, a tantalizing question.

Knowledge of the primary structure of the variants should help us to discriminate among these mechanisms and our grouping of the variants helps us to select particular ones to study in depth. In addition, studies at the nucleic acid level to look for gene rearrangements, such as those shown by Hozumi and Tonegawa (32), have also begun.

Summary

12 variant cell lines producing an $IgG_{2a}(\kappa)$ immunoglobulin derived via different routes from the $IgG_{2b}(\kappa)$ synthesizing MPC 11 were studied. These variants all have the parental MPC 11 idiotype as shown by a radioimmunoassay. A comparison of the variants by charge, peptide maps, and assembly patterns has shown that most of them differ from one another, and some can be grouped.

One group consists of three primary variants generated with two mutagenic agents: these three have almost indistinguishable peptide maps. Two other primary variants which arose in a similar fashion differ markedly from each other and from this group. A second group is comprised of the four secondary variants which arose from two short heavy chain producing primary variants. Other secondary variants and the one spontaneously arising variant cannot be grouped. Possible genetic mechanisms such as translocation, expression of previously silent genes and recombination are discussed.

We thank Dr. Donald Morrison, Professor, Columbia University School of Business, for his assistance in the statistical analysis. We also thank Doctors Richard Stanley and Bernardo Nadal-Ginard, of our Department, for their review of the manuscript and Mrs. Lee Imperato for her capable secretarial assistance.

Received for publication 30 January 1978

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REQUIREMENT FOR HEXOSE, UNRELATED TO ENERGY PROVISION, IN T-CELL-MEDIATED CYTOLYSIS AT THE LETHAL HIT STAGE*

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Although much information has been accumulated on the process of cytolysis by thymus-dependent (T)[1] lymphocytes, the precise biochemical basis for the lytic mechanism is still unclear. The present knowledge of the subject has recently received extensive review (1-3). Three approaches have been adopted for the study of the metabolic processes essential for T-cell-mediated cytolysis. First, experiments have been carried out using support media which have been deprived of primary metabolic substrates. For example, assessment of cytolysis has been performed in glucose-free medium, under anaerobic conditions (4), or in the absence of divalent cations (5). Second, drugs have been used to block cytolysis and the findings have been interpreted in relation to the reported major effects of these drugs on metabolic processes. However, in general no control has been provided to indicate that the observed effect on cytolysis was causally linked to the reported metabolic effects. This approach has been fully reviewed by Martz (2). Finally, competitive inhibitors have been used, with a check on the specificity of their action through reversal of their effect with excess normal substrate. Probably the best example of such an approach has been the recent work of MacDonald (6) using 2-deoxy-D-glucose as an inhibitor of cytolysis, and D-glucose or D-glucose analogs to achieve reversal. These results appear to have formally established a role for glucose or a glucose analog in T-cell-mediated cytolysis. Interestingly, some of MacDonald's results indicated that glucose might not be acting simply as an energy source.

Most previous investigations with drugs, including 2-deoxy-D-glucose, have failed to establish at which stage(s) of cytolysis the agents were acting. T-cell-mediated cytolysis can now be considered as a complex phenomenon including a number of discrete stages (7, 8). The first recognition stage is followed by the "lethal hit" stage during which an irreversible lesion is inflicted upon the target

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* Supported by the Institut National de la Santé et de la Recherche Médicale, and the Délégation Générale à la Recherche Scientifique et Technique grant 76.7.0966.

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[1] *Abbreviations used in this paper:* βNAD, β-nicotinamide adenine dinucleotide; DFBS, dialysed fetal bovine serum; EGTA, ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid; FBS, fetal bovine serum; LDH, lactate-dehydrogenase; LPBS, low phosphate-buffered saline; T lymphocyte, thymus-dependent lymphocyte.

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cell by the effector cell. This is in turn followed by the target cell disintegration stage. Our approach to an analysis of these various stages makes use of a "Ca^{++} pulse" method (3, 9). This method is based on the observation that although the cytolytic process as a whole requires Ca^{++} (5, 9–13), the recognition stage can proceed in the presence of Mg^{++} only (9, 13, 14), and neither Ca^{++} nor Mg^{++} are required at the disintegration stage (5, 8, 10, 15, 16). Ca^{++}, therefore, is necessary only at the lethal hit stage (9, 13). Consequently, the successive addition of Mg^{++}, Ca^{++}, and finally EDTA, provides a simple and convenient way of isolating and studying the lethal hit stage (9). It can be used to investigate the impact of drugs at various stages of the lytic process (3).

In this study we investigated the carbohydrate requirement of T-cell-mediated cytolysis and showed: (*a*) using chemically defined media or the competitive inhibitor 2-deoxy-D-glucose, that glucose or certain glucose analogs are required for the cytolytic process; (*b*) using the Ca^{++} pulse technique and cytochalasin A, which is a selective inhibitor of recognition, that the carbohydrate requirement for cytolysis can be localized at the lethal hit stage; (*c*) that cytolysis can also take place in phosphate-buffered saline plus dialyzed fetal bovine serum without added glucose but even in this support medium cytolysis is blocked by 2-deoxy-D-glucose; (*d*) by studying the three main energy-producing pathways of carbohydrate catabolism, that a major requirement for carbohydrate in T-cell-mediated cytolysis is independent of its capacity to act as an energy source.

Materials and Methods

Media. RPMI-1640 (Gibco Bio-Cult, Glasgow, Scotland) was used as the standard tissue culture medium and as a diluent. Low phosphate-buffered saline (LPBS) was made up as an aqueous solution containing 137 mM NaCl, 2.7 mM KCl, 1.5 mM K_2HPO_4, 3.2 mM Na_2HPO_4 (12 H_2O), 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ (6 H_2O), and 10 mg/l of phenol red. Heat-inactivated (56°C, for 30 min) fetal bovine serum (FBS) (batch 802.42; Eurobio, Paris, France) was used as such or (after dialysis against 100 vol of LPBS with two changes over three 24-h periods) as dialyzed FBS (DFBS).

Chemicals. Chemicals used were D-glucose (Prolabo, Paris, France), 2-deoxy-D-glucose and cytochalasin A (Aldrich Chemical Co., Inc., Milwaukee, Wis.), lactate-dehydrogenase (LDH) from pig heart, and β-nicotinamide adenine dinucleotide (βNAD) grade 1, both from C. F. Boehringer and Sons (Mannheim, W. Germany), and hydrazine hydrate (98% pure; Merck AG, Darmstadt, W. Germany). D-[1-^{14}C]glucose (sp act 4–15 mCi/mM), D-[6-^{14}C]glucose (sp act 3.0 mCi/mM), and 2-deoxy-D-[1-^{14}C]glucose (sp act 59 mCi/mM) were obtained from the Radiochemical Centre (Amersham, England). Sodium ^{51}Cr-chromate (sp act >50 mCi/mg) was obtained from the Commissariat à l'Energie Atomique (Gif-sur-Yvette, France).

Preparation of Sensitized Spleen Cell Populations Containing Cytolytic T Cells. C57BL/6 anti-DBA/2 (H-2^b anti-H-2^d; *b* anti-*d* for short) cell populations containing cytolytic T cells, and the reciprocally sensitized *d* anti-*b* cells were obtained by mixed lymphocyte culture as follows. Mouse splenic responder cells (2×10^7) and irradiated (2,000 rads) allogeneic splenic stimulating cells (2×10^6) were coincubated (37°C in 5% CO_2 atmosphere, for 4–5 days) in an upright plastic flask (Falcon 3013; Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) in a 20-ml vol of RPMI-1640 with 10% FBS, 50 U/ml of penicillin, 50 μg/ml of streptomycin, 2 mM L-glutamine, and 2×10^{-5} M 2-mercaptoethanol. The sensitized cell populations were used for cytolysis and metabolic tests. It is amply documented that the cytolysis obtained under these sensitization conditions and using a short-term assay (see below) is due to T cells (17, 18). It should be stressed that these populations sensitized in vitro contain only a small minority of cytolytic T cells (19) with other T cells, and 30–40% of non-Thy 1-bearing cells (unpublished observations).

Cytolysis Tests. All the assays were done in Cooke microtiter plates with V-shaped wells

(Cooke Laboratory Products, Div. Dynatech Laboratories Inc., Alexandria, Va.), each well receiving 2×10^5 washed, sensitized cells and 10^4 twice-washed P 815 (*d* mastocytoma) cells which had been ^{51}Cr-labeled overnight (20). Upon addition of both types of cells, the microplates were briefly centrifuged (200 *g*, 2 min) to hasten cell-to-cell contacts (20, 21). Incubation was for 4 h at 37°C unless stated otherwise.

In standard assays, target and effector cell populations were washed and resuspended in LPBS and mixed in the cold. Each V-shaped well received successively (*a*) 50 μl of drug and/or nutrient solution, (*b*) 50 μl of the cell mixture (followed by centrifugation), (*c*) after 1 h of incubation at 37°C, 50 μl of 12 mM EDTA to stop further cytolysis (16), (*d*) 5 min afterwards, 50 μl of RPMI-1640 with 10% FBS. The plates were incubated for 3 h at 37°C after the addition of EDTA to allow ^{51}Cr to be released from injured cells. Cell feeding under EDTA cover, after the initial cytolytic stages, allowed exacting media to be tested while the level of spontaneous ^{51}Cr release was kept low.

In Ca^{++} pulse assays (3, 9), target and effector cell populations were washed and resuspended in RPMI-1640 with 1% FBS, 1 mM Mg^{++} (as $MgCl_2$), and 1 mM of ethylene glycol bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA; Sigma Chemical Co., St. Louis, Mo.). The presence of EGTA ensured neutralization of the Ca^{++} contained in RPMI-1640, while Mg^{++} was still available in excess to the cells. The cell populations were mixed in the cold. Each V-shaped well received successively (*a*) 50 μl of the cell mixture (followed by centrifugation), (*b*) after a 40-min incubation at 37°C, 50 μl of Ca^{++} as $CaCl_2$ solution to a final concentration of 1 mM, (*c*) after a further 20-min incubation at 37°C, 50 μl of 12 mM EDTA. Glucose solutions at various concentrations were added as a 50-μl fraction per well either ab initio, or 10 min before the addition of Ca^{++} (prepulse), or just after the addition of EDTA (postpulse). Protocols of individual experiments differing from this general outline are described in Results. The plates were incubated for 3 h at 37°C after the addition of EDTA.

At the end of the incubation period, the plates were centrifuged again, the radioactivity of a 100-μl aliquot from each well was compared with the initial radioactivity of 5×10^3 target cells, and cytolysis was expressed as the averaged percentage of ^{51}Cr released from target cells in triplicate wells.

Measurement of Lactate Production. Sensitized spleen cells were incubated in V-shaped wells of microplates (per well, 4×10^5 cells for 3 h, or 10^6 cells for 1 h, at 37°C in a total volume of 100 μl of LPBS containing the nutrients mentioned in Results). At the end of the incubation, perchloric acid was added to achieve 2% vol/vol in a total volume of 200 μl/well. The plates were centrifuged, and 150-μl supernatant aliquots were transferred to new microplate wells. These aliquots were brought to pH between 6 and 8 (estimated by phenol red in medium) with 20% KOH, and then stored at 4°C for 15 min. The plates were then centrifuged, and 100-μl aliquots were added to tubes containing 100 μl 2% vol/vol βNAD, 1 ml of Tris-hydrazine buffer, pH 9.5, and water to 2.97 ml. The optical density of these solutions was read at 340 nm in 1-cm cuvettes (E^{340} 1 cm). A 30-μl volume of the LDH solution was then added, and the E^{340} 1 cm of solutions was read after 30 min and again after 40 min to check stability. The difference between the initial and final spectrophotometer readings was then subtracted from the medium only blank reading to give ΔE. The lactate present was calculated using the molar extinction coefficient of NADH. Lactate standards were included in experiments. The averaged results for duplicate wells were expressed as nanomoles of lactate produced per 10^6 cells per h.

Measurement of $^{14}CO_2$ *Production from* D-*[1-*^{14}*C]Glucose,* D-*[6-*^{14}*C]Glucose and 2-Deoxy-[1-*^{14}*C]Glucose.* Sensitized spleen cells were incubated in 6×40-mm polystyrene tubes (RT 15; Sterilin, Teddington, Middlesex, England) placed in rubber cap-sealed scintillation vials (Intertechnique, Plaisir, France). Conditions for incubation were the same as for the lactate assay except that 0.025 μCi of [^{14}C]glucose was added to each RT 15 tube. In most experiments, cold glucose was included in excess as specified in Results. At the end of the incubation period, a 200-μl volume of 0.5 M KOH was injected through the rubber seal into each vial outside the RT 15 tube. A 100-μl volume of 30% perchloric acid was then injected into each RT 15 tube. The vials were incubated in a shaking water bath for 1 h at 37°C to allow all CO_2 to be absorbed by the KOH. The RT 15 tubes were then removed and scintillation fluid (5 ml) was added directly to each vial. The amount of CO_2 produced from carbon 1 or 6 of D-glucose, termed 1-C.CO_2 and 6-C.CO_2 respectively, was estimated as:

$$\frac{\text{counts per minute measured}}{\text{counts per minute added}} \times \text{nanomoles D-glucose added.}$$

(This made no allowance for glucose recycled from the pentose cycle and diluting D-[1-^{14}C]glucose, but in the experiments quoted, the total glucose added to cultures was sufficiently large to make this a minor error.) The main purpose of the metabolic measurements in this study was to gain information about the doses of drugs which effectively stop and the levels of nutrients which effectively support energy production by the three major glycolytic pathways. Only rough estimates of absolute traffic could be derived from the data presented. Methods of calculating the relative activity of the main glucose utilization pathways were discussed in detail by Katz and Wood (22). Measurements of 1-C.CO_2, 6-C.CO_2, and lactate production were expressed as nmol/10^6 cells/h.

Repetition of Experiments. All cytolysis and metabolic experiments reported were carried out at least three times yielding similar results. All nutrient and drug solutions were prepared fresh for each assay.

Results

The Relative Contribution of Different Metabolic Pathways to Energy Production in Sensitized Spleen Cells at Different Glucose Concentrations. To set a basis for the studies of carbohydrate utilization during cytolysis, the relative contribution of the main metabolic pathways of energy production in sensitized spleen cells was examined. Although the metabolic activity of cytolytic T cells is not necessarily reflected by the sum activity of 5-day one-way mixed lymphocyte cultures, it was considered that these measurements would provide a better indication of the active dose range of metabolic inhibitors than could be obtained from reports of previous studies on totally different cell populations. Sensitized spleen cells (10^6) were incubated for 3 h in a total volume of 100 μl of LPBS in the presence of different concentrations of D-glucose, after which the amount of metabolites produced was assessed (Table I).

Lactate production of >4 mM glucose (800 nmol/culture) was constant, and the relative contribution of the pentose cycle to lactate production was well under 5%. Lactate production was markedly less when limiting glucose concentrations were used, with a corresponding increase of the relative importance of the pentose cycle and Krebs cycle. Experiments using lower concentrations of glucose in 100-μl cultures showed that lactate production was below the level of sensitivity of the assay used at $\cong 0.05$ mM glucose. This value is useful for interpreting experiments (described below) on the supportive role of DFBS in cytolysis.

The Carbohydrate Requirement of T-Cell-Mediated Cytolysis. Two lines of evidence strongly suggested that D-glucose (or one of its metabolites or analogs) was required for T-cell-mediated cytolysis. First, good levels of specific cytolysis could be produced in LPBS plus D-glucose, but there was no detectable cytolysis in LPBS alone (Fig. 1). Second, 2-deoxy-D-glucose was able to competitively inhibit cytolysis when in excess over glucose (Fig. 2). It was possible to delay the addition of glucose for 40 min in cultures initiated in LPBS and still get cytolysis (Table II, group 1). This showed that glucose deprivation was not immediately lethal for the cytolytic T cells. Furthermore, the blocking effect of 2-deoxy-D-glucose added ab initio could be reversed by the addition of excess glucose 40 min later (Fig. 3). This indicated a complete reversibility by glucose of the 2-deoxy-D-glucose block of cytolysis. Taken together, these results

TABLE I

Assessment of 1-C.CO$_2$, 6-C.CO$_2$, and Net Lactate Production by Sensitized Spleen Cells Cultured in LPBS Plus Varying Concentrations of Glucose

Production of*	Glucose concentration (mM)					
	16	8	4	2	1	0.5
Lactate	30	30	29	25	18	7
1-C.CO$_2$	3.9	4.5	6.6	6.2	5.9	4.6
6-C.CO$_2$	0.24	0.24	0.31	0.31	0.26	0.24

* Results are expressed in nmol/10^6 cells/h.

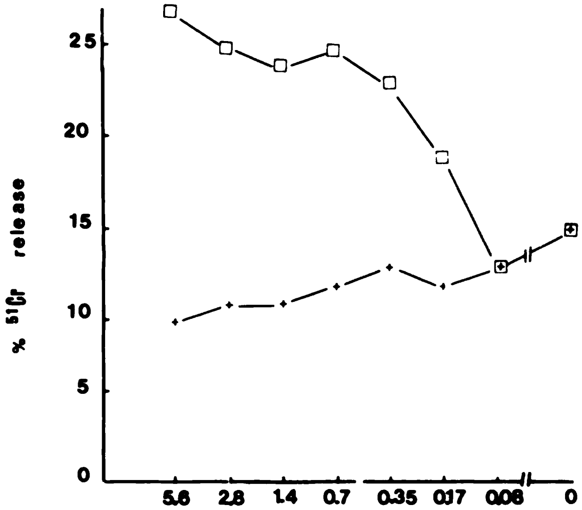

FIG. 1. Concentration of D-glucose able to support T-cell-mediated cytolysis in LPBS. Each culture contained 10^4 target cells (P 815 of *H-2^d* origin) and 2 × 10^5 effector cells. Cytolysis mediated by *b* anti-*d* (□) and *d* anti-*b* (+) effector cells.

showed that glucose was required to support cytolysis and not just to ensure cytolytic cell survival.

The Stage of the Cytolytic Process Where Glucose is Required. Calcium pulse experiments were carried out to see if glucose was required for the Ca^{++}-dependent lethal hit stage of cytolysis. 2-deoxy-D-glucose was added to glucose-containing effector cell-target cell mixtures, either ab initio, 10 min before the Ca^{++} pulse, or just after the Ca^{++} pulse. Block occurred with prepulse but not with postpulse addition of 2-deoxy-D-glucose (Fig. 4). This type of experiment indicated that glucose was required at the lethal hit stage but did not give information about possible glucose requirements at the recognition stage. To investigate this, the drug cytochalasin A was used. At appropriate concentrations, this agent inhibits recognition but not the Ca^{++}-dependent lethal hit stage (23).[2] Cell mixtures were set up in LPBS only, and glucose was added either before or after the addition of cytochalasin A. The detailed protocol of the

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[2] P. Golstein, C. Foa, and I. C. M. MacLennan. 1978. Mechanism of T-cell-mediated cytolysis: the differential impact of cytochalasins at the recognition and lethal hit stages. *Eur. J. Immunol.* In press.

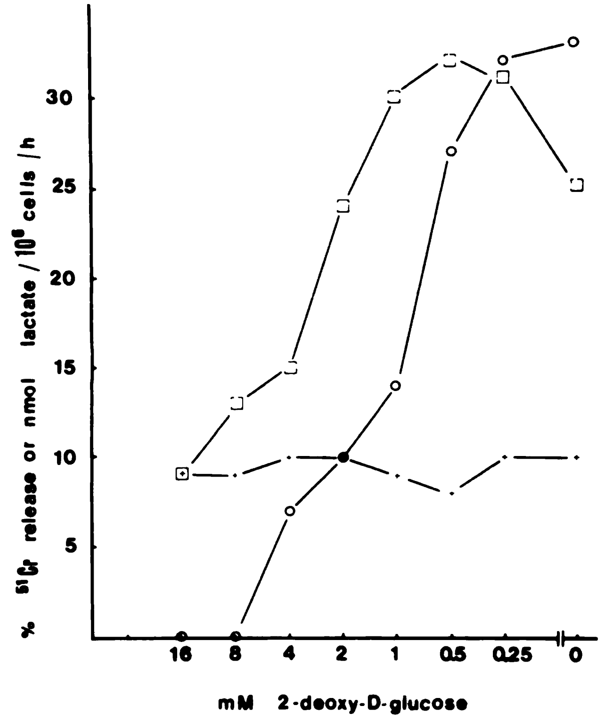

FIG. 2. Concentration of 2-deoxy-D-glucose required to inhibit (*a*) specific T-cell-mediated cytolysis; (*b*) lactate production by sensitized T cells. Cytolysis mediated by *b* anti-*d* (□) and *d* anti-*b* (+) effector cells. (○), Lactate production by *b* anti-*d* cells. The basic culture medium was LPBS + 3 mM D-glucose.

TABLE II

Lack of Glucose Requirement for the Cytochalasin A-Dependent Stage of Recognition

Group	Time (min) of culture addition*						Cytolysis of *d* target cells by the following effector cells‡	
	Pre 0	0	30	40	60	65	*b* anti-*d*	*d* anti-*b*
1	LPBS	Cells in LPBS	LPBS	Glucose	EDTA	RPMI 10% FBS	28.8	16.2
2	LPBS	"	Cytochalasin A	"	"	"	25.3	14.9
3	Cytochalasin A	"	LPBS	"	"	"	18.2	15.3
4	LPBS	"	LPBS	LPBS	"	"	17.1	16.7
5	Glucose	"	Cytochalasin A	"	"	"	26.2	16.1

* Additions at time pre 0, 0, 30, and 40 min in 25 μl. Additions at 60 and 65 min in 50 μl. No serum additive was used before 65 min. Cultures were harvested at 4 h.

‡ Cytolysis expressed as percent ^{51}Cr release (means of quadruplicate cultures).

experiment and its results are shown in Table II. A comparison of groups 2 and 5 in particular indicated that the cytochalasin A-inhibitable step did not require glucose. Taken together, these experiments showed that D-glucose was required during the lethal hit stage of cytolysis, but was not required at either the cytochalasin A-inhibitable recognition stage, nor the ^{51}Cr release stage.

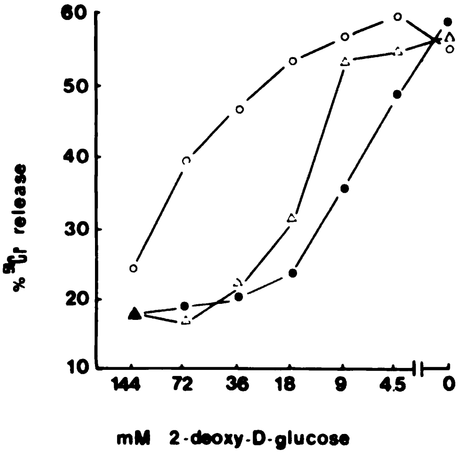

FIG. 3. Ability of D-glucose to reverse the inhibition of cytotoxicity induced by 2-deoxy-D-glucose. All cultures were set up with 10^4 P 815 and 2×10^5 *b* anti-*d* cells in RPMI-1640 (11.5 mM D-glucose) with 1% FBS plus the dilutions of 2-deoxy-D-glucose shown. The cultures were then centrifuged and incubated for 40 min at 37°C before glucose supplements were added to the culture: no supplementary D-glucose (●), 18 mM D-glucose (△), 72 mM glucose (○). Cultures were harvested 3 h later.

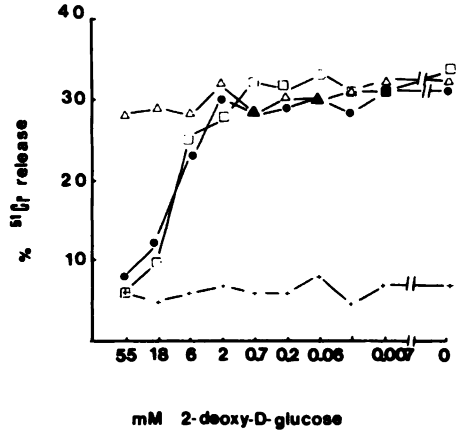

FIG. 4. The stage of T-cell-mediated cytolysis inhibited by 2-deoxy-D-glucose. Cultures of 10^4 P 815 and 2×10^5 effector cells were set up in RPMI-1640 + 1% FBS, 1 mM EGTA, and 1.5 mM Mg^{++} to allow binding without lysis. After 40 min, Ca^{++} was added (final concentration 1 mM) to initiate the lethal hit. 20 min later, EDTA was added (final concentration 3 mM). Cultures were harvested 3 h later. (△), *b* anti-*d* effector cells, 2-deoxy-D-glucose added after EDTA; (□), *b* anti-*d* effector cells, 2-deoxy-D-glucose added ab initio; (●), *b* anti-*d* effector cells, 2-deoxy-D-glucose added before Ca^{++} pulse; (+), *d* anti-*b* effector cells, 2-deoxy-D-glucose added ab initio.

Three Lines of Evidence Suggesting that the Role of D-Glucose in T-Cell-Mediated Cytolysis Is Unrelated to Its Capacity to Provide Energy

2-DEOXY-D-GLUCOSE, WHICH BLOCKS CYTOLYSIS, CAN ACT AS AN ENERGY SOURCE. The experiments exemplified in Fig. 2 clearly showed the efficiency of 2-deoxy-

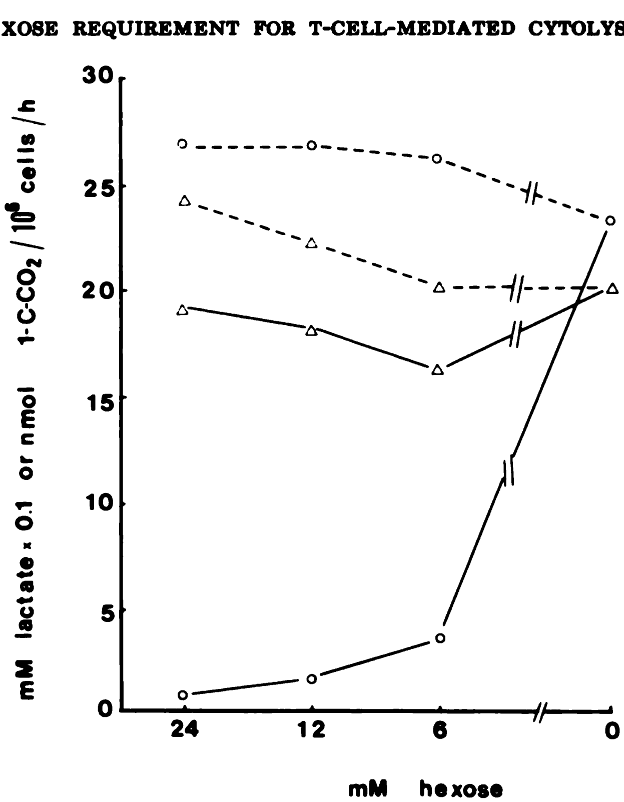

FIG. 5. Failure of 2-deoxy-D-glucose to inhibit 1-C.CO$_2$ production from the 1-C atom of glucose by *b* anti-*d* effector cells. Medium used was 5 mM D-glucose in LPBS. 1-C.CO$_2$ production (△——△), net lactate production (O——O) with added 2-deoxy-D-glucose; 1-C.CO$_2$ production (△- - - △), net lactate production (O- - -O) with added D-glucose.

D-glucose at blocking lactate production. However, as this agent does not block the cytochrome system (24), it could be a source of energy via the pentose cycle, provided that it did not prevent the reduction of NADP in the first or second enzymatic steps of this cycle. This was investigated in two ways. First, the effect of 2-deoxy-D-glucose on $^{14}CO_2$ production from D-[1-^{14}C]glucose by sensitized spleen cells was assessed. Fig. 5 shows that 2-deoxy-D-glucose, even at concentrations which inhibited lactate formation, did not significantly inhibit 1-C.CO$_2$ production from glucose. The second set of experiments tested the relative amounts of $^{14}CO_2$ produced from D-[1-^{14}C]glucose and 2-deoxy-D-[1-^{14}C]-glucose. These confirmed the previous experiments in that equivalent amounts of $^{14}CO_2$ were produced from both radiochemicals (Table III). This shows that 2-deoxy-D-glucose could be processed through the first two enzymatic steps of the pentose cycle and thus provide a potential energy source in the form of reduced NADP, which if transported to mitochondria, could be oxidized to yield ATP.

OTHER SUBSTRATES FOR ENERGY METABOLISM DO NOT SUPPORT CYTOLYSIS. A number of known substrates for energy metabolism were tested for their capacity to support T-cell-mediated cytolysis. These were glycerol, pyruvate, and the following glycogenic amino acids: alanine, arginine, asparagine, glycine, histidine, phenylalanine, serine, and valine. They were assessed at

TABLE III

Ability of Sensitized Spleen Cells to Produce 1-C.CO_2 from 2-Deoxy-D-Glucose in the pentose cycle

Culture conditions*			Production of 1-C.$^{14}CO_2$‡
2-Deoxy-[1-^{14}C]glucose	[1-^{14}C]Glucose	D-Glucose	
μM	*μM*	*mM*	
0.5	–	5.6	0.96
–	7.5	5.6	0.74

* Sensitized cells are cultured in RPMI-1640 with either [1-^{14}C]D-glucose or 2-deoxy-[1-^{14}C]D-glucose added.

‡ Expressed as nmol $CO_2/10^6$ cells/h.

TABLE IV

Ability of Exogenous Pyruvate to Compete with Pyruvate Produced by Glycolysis

Release of*	Pyruvate concentration (mM)			
	10	3.3	1.1	0
		cpm		
6-C.$^{14}CO_2$	65	126	210	626
1-C.$^{14}CO_2$	4,100	5,782	6,930	3,811

* Cultures were set up in LPBS plus 0.5 mM glucose including either [1-^{14}C]D-glucose or [6-^{14}C]D-glucose. The radioactive CO_2 released by the cultures was measured and is given here as counts per min (cpm). The rise in 1-C.CO_2 production associated with pyruvate is considered in the discussion.

final concentrations of 10 mM in LPBS and three twofold dilutions from this. Positive control groups with D-glucose were included. None of these substrates permitted significant T-cell-mediated cytolysis, whereas D-glucose supported significant cytolysis in each experiment (not shown). Experiments were set up with pyruvate or a mixture of glycogenic amino acids to see if the agents could be used as substrates for aerobic metabolism. Pyruvate strongly inhibited 6-$C^{14}CO_2$ production from glucose (Table IV), indicating that exogenous pyruvate could compete with cell-derived pyruvate as a substrate for energy metabolism. The ability of the glycogenic amino acids to act in this way was not clear from these experiments (not shown). An overall conclusion from these and the 2-deoxy-D-glucose experiments is that provision of energy without glucose or DFBS (see below) is not sufficient to support T-cell-mediated cytolysis.

DIALYZED FBS CAN SUPPORT T-CELL-MEDIATED CYTOLYSIS IN THE ABSENCE OF DETECTABLE LACTATE PRODUCTION. Experiments were set up to see if LPBS enriched with DFBS only, without added glucose, could support cytolysis. It was found (Fig. 6) that as little as 0.03% DFBS was able to do so. This result immediately posed serious questions in relation to the glucose dependence of cytolysis, for (*a*) thoroughly dialyzed FBS should contain no free glucose, and (*b*) there is insufficient nondialyzable carbohydrate in FBS for the agent to be

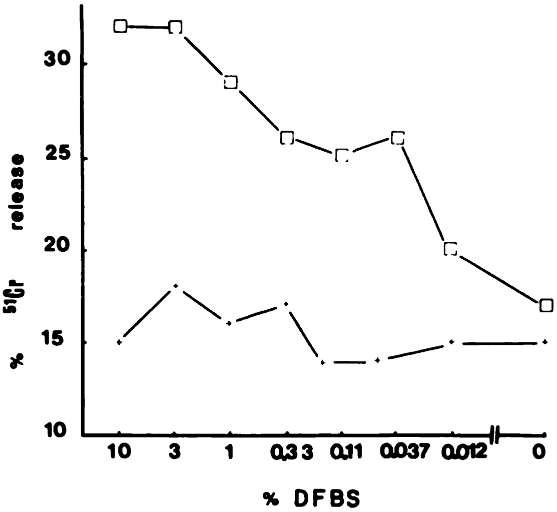

FIG. 6. Concentration of DFBS able to support T-cell-mediated cytolysis in LPBS. Each culture contained 10^4 P 815 target cells and 2 × 10^5 *b* anti-*d* (□), or *d* anti-*b* (+) effector cells.

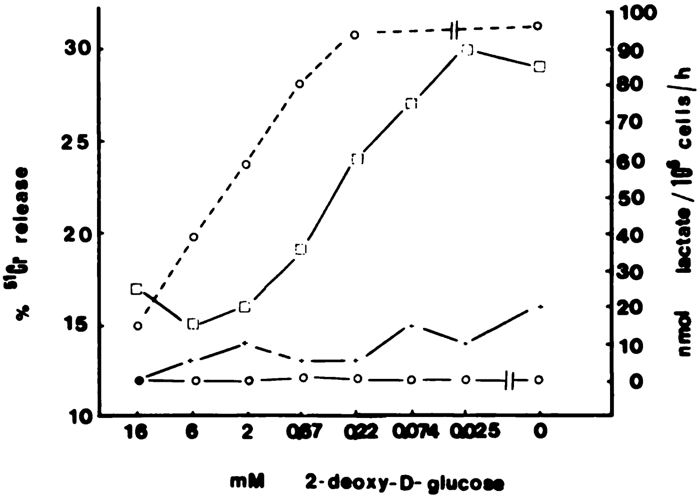

FIG. 7. Ability of 2-deoxy-D-glucose to inhibit T-cell-mediated cytolysis in LPBS + 5% DFBS and failure of 5% DFBS to support net lactate accumulation by effectors. Cytolysis by *b* anti-*d* (□), or *d* anti-*b* (+) effector cells. Lactate production by *b* anti-*d* cells in LPBS + 5% DFBS (O——O), or in LPBS + 3 mM glucose (O- - -O).

acting simply as a glucose source. Even if the mean carbohydrate content were 10% of serum proteins (100 g/liter) and all were available hexose, there would be only 0.017 mM hexose in 0.03% DFBS. This is well below the minimum glucose concentration able to support cytolysis in LPBS (Fig. 2). However, carbohydrates do appear to be essential in DFBS-supported cytolysis, since this was blocked by 2-deoxy-D-glucose (Fig. 7). Perhaps even more interestingly, when incubated in LPBS plus 5% DFBS (an excess of >100-fold over the

TABLE V

Level of 2-Deoxy-D-Glucose Required to Inhibit Cytolysis in LPBS Plus Various Amounts of DFBS

2-Deoxy-D-glucose	DFBS (%)			
	25	8	2	0.7
mM				
10	17*	15	16	18
3.3	17	16	17	17
1.1	22	22	21	20
0	30	33	33	33

* Cytolysis expressed as percent ^{51}Cr release from 10^4 *d* target cells in the presence of 2×10^5 *b* anti-*d*-sensitized spleen cells. Percent ^{51}Cr release in presence of *d* anti-*b* cells is not shown but was in the range of that seen with *b* anti-*d* cells plus 10 mM 2-deoxy-D-glucose.

TABLE VI

Synergism between Glucose and DFBS in Overcoming the Block on Cytolysis Induced by 2-Deoxy-D-Glucose

2-Deoxy-D-glucose (mM)	LPBS + 10% DFBS				LPBS + 6 mM glucose			
	16	8	4	0	16	8	4	0
D-Glucose supplement *mM*								
0	4/4*	5/4	7/4	20/4	7/6	8/7	10/7	22/8
8	17/5	22/5	24/6	20/4	10/6	14/7	17/8	22/6
16	23/5	24/4	25/5	22/5	14/7	16/7	20/7	24/10
32	24/5	23/5	23/5	24/6	19/9	21/8	21/8	26/12

* Cytolysis expressed as percent ^{51}Cr release from 10^4 *d* target cells in presence of 2×10^5 *b* anti-*d*/*d* anti-*b* effector cells.

minimum DFBS concentration required to support cytolysis), sensitized spleen cells did not produce measurable levels of lactate (Fig. 7). These experiments led us to two conclusions. First, whereas glucose did not seem to be involved in DFBS-supported cytolysis, as demonstrated by the quantitative considerations above, and by the absence of lactate production, clearly something closely related to glucose must be involved because of the inhibitory effect of 2-deoxy-D-glucose. Second, T-cell-mediated cytolysis could take place in the absence of detectable lactate production.

Does DFBS Directly Provide a Necessary Substrate for Cytolysis? To test this, the dose of 2-deoxy-D-glucose required to block cytolysis in LPBS plus dilutions of DFBS was assessed. The results of such an experiment are shown in Table V. The concentration of 2-deoxy-D-glucose required to inhibit cytolysis was found to be independent of the amount of DFBS added to cultures. Such a result would seem to indicate that 2-deoxy-D-glucose is not competing directly with a substrate in DFBS.

Synergism between D-Glucose and DFBS. A consistent observation has been that it was far more difficult to inhibit cytolysis with 2-deoxy-D-glucose when both DFBS and D-glucose were present than when either supportive agent was present alone. A typical experiment is illustrated in Table VI. This synergism is marked at DFBS concentrations >5%, but is not obvious with 1% DFBS in LPBS. This observation reinforces the conclusions drawn above that DFBS is acting neither as a direct glucose source nor simply as an agent for mobilizing glucose.

Discussion

Now that it is possible to isolate different stages of T-cell-mediated cytolysis, the task of unravelling the biochemical basis of the process has become more approachable. This is exemplified in our study, which suggests an absolute requirement for glucose or a glucose derivative during cytolysis, and also indicates that this requirement is confined to the calcium-dependent lethal hit stage of cytolysis.

First, is D-glucose really necessary for cytolysis? It seems from the experiments showing specific T-cell-mediated cytotoxicity in LPBS plus D-glucose, but not in LPBS alone, that D-glucose is able to satisfy some molecular requirement for cytolysis. But these experiments do not tell us whether D-glucose is obligatory, or simply a substrate for synthesis which could be replaced by other molecules. At first the experiments showing lysis in LPBS with <0.1% DFBS appeared to indicate that D-glucose was not obligatory. But the finding that 2-deoxy-D-glucose blocked cytolysis in this support medium returned the balance in favor of glucose involvement. However, we know that cytolysis only proceeds in LPBS plus D-glucose when the D-glucose concentration is >0.1 mM, and that there is insufficient carbohydrate in 0.04% dialysed FBS in LPBS to provide this level of D-glucose. Also, the experiments showing no lysis in LPBS plus amino acids or pyruvate make it improbable that hexose could be synthesized from DFBS protein catabolism. This seems to leave three possible explanations for the mechanism of action of DFBS: (*a*) that it mobilizes carbohydrate from the cells, (*b*) that DFBS provides a carbohydrate molecule which is more efficient than glucose, (*c*) that DFBS increases the efficiency of cytolysis in some way, so as to reduce the requirement for glucose, for instance by bringing about increased cell-cell binding. The second of these possibilities seems unlikely because the concentration of 2-deoxy-D-glucose needed to block cytolysis in LPBS plus dialyzed FBS is independent of the concentration of dialyzed FBS (Table V). This observation is, however, compatible with explanation (*a*), (*c*), or a combination of both explanations.

Another indirect line of evidence compatible with D-glucose requirement at the lethal hit stage comes from studies with cytochalasins. Both cytochalasin A and B will block cytolysis when added to cultures ab initio, but only cytochalasin B inhibits when added after recognition and before the calcium pulse.[2] It is interesting that cytochalasin B, but not cytochalasin A blocks glucose utilization by sensitized spleen cells (25).[2] Perhaps the most convincing argument in favor of an absolute requirement for D-glucose or a D-glucose analog in cytolysis is the fact that 2-deoxy-D-glucose reversibly blocks cytolysis in complete culture medium (ref. 6 and Fig. 3). The selectivity of the reaction is further emphasized by MacDonald's observations (6) that many other hexoses and pyruvate fail to reverse the 2-deoxy-D-glucose block of cytolysis. Galactosamine, galactose, fucose, and fructose all failed to reverse the block. However, it is of great interest that 5-thio-D-glucose and mannose could reverse this inhibition. The efficiency of 5-thio-D-glucose in competing with 2-deoxy-D-glucose, combined with the failure of trioses to support cytolysis, seems to indicate that D-glucose is used in the cytolytic process either as intact hexose or an early modification of hexose.

This argument implies that the role of D-glucose at the lethal hit stage is unrelated to the capacity of this compound to act as an energy source. The experiments shown in Table IV indicate that pyruvate is a perfectly good source of energy (ATP generation) in sensitized spleen cells as a whole. There is no good reason to suppose that the cytolytic T cells in this cell mixture should not also be able to derive energy from pyruvate by aerobic metabolism. However, pyruvate does not support cytolysis. Also, the aerobic metabolic pathways are not blocked by 2-deoxy-D-glucose (24) which is able to inhibit cytolysis. These results indicate that energy provision alone is not sufficient for cytolysis to proceed in simple salt solutions.

Is energy required at all for T-cell-mediated cytolysis? Most published work on this question involves the use of metabolic inhibitors. Iodoacetate has been shown to block T-cell-mediated cytolysis at $\cong 10^{-4}$ M (3, 26). This concentration of drug does block lactate production in sensitized spleen cells, but fails to inhibit aerobic metabolism.[3] Table I clearly indicates that Krebs cycle activity becomes the major source of energy at low glucose concentrations, so it is hard to conclude that iodoacetate is acting by blocking energy supplies necessary for cytolysis. This conclusion is reinforced by the observation that pyruvate fails to reverse the effect of this drug (3). These experiments as well as those with DFBS (Fig. 7) indicate that cytolysis can occur where net lactate accumulation is unmeasurable. Sodium azide in normal culture medium causes 50% block of cytolysis at $\cong 30$ mM (3, 26–28). This concentration of azide is approximately ten times that required to cause an equivalent inhibition of 6-C CO_2 production from glucose, but it is insufficient to inhibit glycolysis.[3] However, in the absence of exogenous glucose but in the presence of DFBS, i.e. under conditions that we found led to no detectable accumulation of lactate, cytolysis was blocked by concentrations of azide equivalent to those required to inhibit the terminal cytochrome system (4), which seems to indicate a minimal energy requirement for cytolysis.

The finding that 2-deoxy-D-glucose does not inhibit the formation of reduced NADP by the first two stages of the pentose cycle is of interest. Granulocytes undergo a burst of pentose-cycle activity during phagocytosis (29). It has been suggested that reduced NADP plays a vital role in the production of bactericidal products, particularly hydrogen peroxide (30). However, Michl et al. (31, 32) have shown that phagocytosis, but not binding of C_3 and IgG-coated erythrocytes by mouse peritoneal macrophages, is blocked by 2-deoxy-D-glucose. This effect appeared to be independent of ATP production. These authors showed that 2-deoxy-D-glucose did not inhibit opsonin-independent phagocytosis of latex particles, which suggests that this block may be interfering with a specific recognition process. It will be interesting to see if comparative studies show any close similarities between this block on phagocytes compared with that seen on T-cell-mediated cytolysis. Our data do not exclude a role for NADPH, but they do show that the 2-deoxy-D-glucose inhibition is not due to prevention of reduction of this coenzyme. NADPH could potentially act as an energy source if it were transported to mitochondria. However, there is no evidence that this occurs in cytotoxic T cells. Indeed, the experiment shown in Table IV suggests that NADPH may be accumulating in the cytoplasm. This experiment shows that pyruvate potentiates the production of 1-C.CO_2 from glucose, and this may be because pyruvate can oxidize NADPH either by combination with CO_2 to produce malate (33) or by reduction of pyruvate to lactate (34). The removal of NADPH is important, as this substance acts as a feedback inhibitor of glucose-6-phosphate oxidation by glucose-6-phosphate dehydrogenase (35).

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[3] I. C. M. MacLennan and P. Golstein. 1978. Manuscript in preparation.

How then is D-glucose acting in the cytolytic process, and how is 2-deoxy-D-glucose interfering with it? One of the actions of 2-deoxy-D-glucose which has recently received much attention has been its effect on the glycosylation of proteins. Eagon and Heath (36) showed that a K light chain made by BALB/c plasmacytoma cells cultured in 2-deoxy-D-glucose was synthesised and secreted without its carbohydrate side chain. These authors showed that 2-deoxy-D-glucose inhibited the incorporation of glucosamine, mannose, and galactose, which seems to indicate that this inhibitor has a broad range of action on hexose incorporation with glycoproteins. Hughes et al. (37) demonstrated a similar broad specificity of action of 2-deoxy-D-glucose in inhibiting protein glycosylation by hamster kidney cells. However, Meager et al. (38), using the same system, showed less inhibition of mannose incorporation than glucosamine and galactose. Mannose, as well as glucose, was found by MacDonald to reverse the 2-deoxy-D-glucose block of T-cell-mediated cytolysis (6). It has been shown that 2-deoxy-D-glucose interferes with virus-induced cell fusion (39). This effect is also reversed by mannose and glucose. Thus, the processes involved in these cases seem to exhibit similar specificity for glucose, mannose, and 2-deoxy-D-glucose.

It has been suggested that the impairment of cell fusion mentioned above may be due to a lack of replacement of shed surface glycoproteins, which is in line with a glycoprotein requirement for virus-induced cell fusion. For T-cell-mediated cytolysis at the lethal hit stage, a glycoprotein may also be required, synthesized by glycosylation of a preformed protein either in permanence or only upon a post-recognition activation process. This glycosylation step could be the one which is inhibited by 2-deoxy-D-glucose. Alternatively, this agent could inhibit another hexose-specific molecular recognition step occurring during the lethal hit. Whatever the proposed mechanism, it would have to fit in with the alterations of hexose concentration requirement observed in the presence of FBS.

Models such as these are of some help in designing further experiments to probe the mechanism of the lethal hit in T-cell-mediated cytolysis. These models must now take into account not only the fact that this stage of the lytic process is dependent on calcium ions and is sensitive to agents such as azide and iodoacetate, but also that it has a requirement for hexose which is independent of its capacity to act as an energy source.

Summary

The requirement for D-glucose in T-cell-mediated cytolysis was studied using mouse spleen cells sensitized against alloantigens in vitro. Glucose was required for cytolysis: (*a*) cytolysis proceeded in a simple buffered salt solution containing Ca^{++} and Mg^{++} (low phosphate-buffered saline, LPBS) in the presence but not in the absence of added glucose; (*b*) 2-deoxy-D-glucose blocked cytolysis. The block by this agent was overcome by excess glucose added as late as 40 min after the inhibitor. This block was not due to inhibition of NADP reduction, since 2-deoxy-D-glucose failed to interfere with the rate of CO_2 production by the pentose cycle which we found to be of significant activity in sensitized spleen cells; (*c*) dialyzed fetal bovine serum (DFBS) in LPBS supported cytolysis in the absence of added glucose. However, 2-deoxy-D-glucose was also inhibitory under these conditions, suggesting that carbohydrate was required here as well. Further results supported the conclusion that DFBS was not acting as a direct source of the required carbohydrate.

The relationship between cytolysis, glucose requirement, and provision of energy was studied. As little as 0.1 mM D-glucose in LPBS supported cytolysis. At this glucose concentration, there was no measurable accumulation of lactate in sensitized spleen cells, but Krebs cycle activity was detectable. In 3 mM glucose or above, the range covered by standard tissue culture media, anaerobic glycolysis became a major source of energy in sensitized spleen cells. Consequently, it appears that in standard tissue culture medium, effector cells can generate sufficient energy for cytolysis either by aerobic or anaerobic metabolism. However, the addition of an energy source alone in the absence of glucose was insufficient to support cytolysis in LPBS. Pyruvate in LPBS did not support cytolysis but was shown to be a good substrate for aerobic metabolism in sensitized spleen cells. Glycogenic amino acids and glycerol also failed to support cytolysis.

The stage of cytolysis at which glucose is required was investigated. Glucose was necessary for the calcium-dependent lethal hit phase, but not for the cytochalasin A-blockable recognition stage, nor for ^{51}Cr release from injured target cells. Models for the lethal hit process are discussed, which are compatible with the observed requirement for certain hexoses unrelated to their capacity to serve as sources of energy.

We thank D. Williamson and Marian Stubbs from the Metabolic Research Unit at Oxford for their advice on metabolic aspects of this study, and Marie-Françoise Luciani for her excellent technical assistance.

Received for publication 10 January 1978.

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EFFECT OF CASTRATION AND SEX HORMONE TREATMENT ON SURVIVAL, ANTI-NUCLEIC ACID ANTIBODIES, AND GLOMERULONEPHRITIS IN NZB/NZW F_1 MICE*

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NZB/NZW F_1 (B/W)[1] mice are an experimental model for systemic lupus erythematosus. They spontaneously develop an autoimmune disease characterized by the formation of antibodies to nucleic acids and the development of a fatal immune complex glomerulonephritis. There is an accelerated expression of the disorder in female animals (1). Genetic (2), immunologic (3), and viral (4) factors are involved in pathogenesis. A disorder of immunologic regulation related to abnormalities in T-cell differentiation and function may represent a fundamental derangement in these animals (5).

The accelerated disease in female B/W mice is associated with an earlier appearance of IgG antibodies to DNA and polyadenylic acid (Poly A) (6), an earlier onset and greater severity of immune complex nephritis, and a markedly decreased incidence of survival beyond 12 mo (7). Males develop these abnormalities later in life. Prepubertal castration of male B/W mice caused an essentially female pattern of disease with 100% mortality at 11 mo (8). By contrast, prepubertal castration of female B/W mice had no effect on mortality, although it markedly reduced the development of IgG antibodies to Poly A. In a preliminary experiment, the administration of androgen to castrated females resulted in prolonged survival (8).

These results suggest that sex hormones modulate the expression of autoimmunity in B/W mice, with androgens exerting a protective influence. Androgens delay the switch from IgM to IgG antibodies to nucleic acids (8), a differentiation event which is generally thymic-dependent (9). In B/W mice, the development of IgG antibodies to Poly A is abrogated by neonatal thymectomy (10). These findings suggest that sex hormones modulate autoimmunity by acting on thymic-dependent regulatory mechanisms.

In the present report, prepubertal castration of B/W mice was accompanied by the sustained administration of sex hormones. The results demonstrate that androgens and estrogens have opposing effects on disease expression and survival. The possible therapeutic use of male hormone is suggested. In addition, experiments combining castration with neonatal thymectomy suggest

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* Supported by the Medical Research Service of the Veterans Administration, by U. S. Public Health Service grant AM 16140, by a grant from the Kroc Foundation, and a contract from the State of California.

[1] *Abbreviations used in this paper*: B/W mice, (NZB × NZW) hybrid mice; PBS, phosphate-buffered saline; Poly A, polyadenylic acid.

that sex hormones require the thymus in order to express their immunologic effects.

Materials and Methods

Mice. B/W mice were derived from our colony at the University of California Vivarium, San Francisco, Calif., and they were maintained at the Fort Miley Veterans Administration Hospital.

Four separate experimental protocols were followed: (*a*) prepubertal castration combined with hormone implantation at 2 wk of age, (*b*) retreatment with a second androgen implant at 9 mo of age, (*c*) androgen treatment of intact males at 8 mo of age, and (*d*) combined neonatal thymectomy and prepubertal castration of males.

In the first protocol, there were 11 sham-operated males, 32 receiving androgen, and 21 receiving estrogen. There were 13 sham-operated females, 14 receiving androgen, and 17 receiving estrogen. In the retreatment protocol, 10 of the original 32 castrated and androgen-treated males were used. Five mice received a second implant containing androgen, and five received an empty implant. As for the females, 6 of the original 14 that were castrated and androgen-treated at 2 wk received a second implant containing androgen at 9 mo. These were compared with five surviving females from the original sham group.

In the third protocol, 9 intact males served as shams and 14 received an androgen implant for the first time. All animals were 8 mo of age. In the fourth experiment, seven male mice were subjected to combined thymectomy and castration procedures, and 10 were sham-operated.

Operative Techniques

Animals were subjected to castration, or they were sham-operated at 2 wk of age. They were anesthetized by intraperitoneal injection of Nembutal.

OOPHORECTOMY. A midline incision was made over the abdomen. Ovaries were identified and removed using electrocautery. The incision was closed using 6-0 silk suture material.

ORCHIECTOMY. Testes were delivered through a scrotal incision. The vas deferens and spermatic vessels were transected using electrocautery, and testes and epididymis were removed. Skin was sutured using 6-0 silk suture material.

THYMECTOMY. (Controlled suction technique) Thymectomy was carried out at 2 days of age under a dissecting microscope at a magnification of 1.6 ×. At this magnification, the entire mediastinal cavity was in the field of vision. The sternal incision was extended from the manubrium down to the sixth rib. We gently mobilized the thymic lobes by disrupting the vascular and connective tissue attachments. To achieve adequate control of suction intensity, a 3-mm hole was made in a 5-cm tuberculin syringe barrel which was interspersed between the curved pipette and the rubber tubing connected to the vacuum outlet. Each thymic lobe was engaged by the pipette at the lower pole and gently teased off. The entire removal process was visualized under the microscope. The mediastinal cavity was left empty.

Sham Operation. In age-matched sham-operated controls, ovaries, testes, or thymic lobes were identified, but left intact. Experimental animals were distinguished from sham-operated mice by tail clipping or ear tagging. To minimize maternal neglect, the surgical incision and maternal nasal orifices were painted with parlodion-gentian violet solution.

Hormone Replacement. To achieve a maintained hormone replacement in castrated animals, a 2-cm Silastic tube containing 6–7 mg of either estradiol-17-β or 5-α-dihydrotestosterone powder was implanted subcutaneously immediately after the castration procedure. This treatment resulted in prompt establishment of an adequate hormone level reached within 12 h, which persisted for at least 3 mo. Sham-operated mice received empty implants.

Observation. Mice were observed weekly. Mortality in experimental and control groups was tabulated in 2 × 2 contingency tables, and evaluated by Chi square analysis using Yates modification.

Determination of Serum Testosterone and Dihydrotestosterone Levels. Serum samples from sham-operated or castrated and androgen-treated male and female mice were pooled separately. Serum testosterone and dihydrotestosterone were measured using a radioimmunoassay similar to that described by Abraham (11). Both androgens were purified before assay by celite chromatography and [^{3}H]testosterone was added to 0.5-ml serum samples before extraction with ether to correct results for losses. The antisera used were prepared against testosterone coupled to bovine serum albumin by the succinate ester, and they showed 66% cross-reactivity with dihydrotestosterone.

Histopathology. At ages ranging from 4 to 11 mo, randomly selected mice from each group were sacrificed. Their kidneys were removed and studied by light and electron microscopy, and by immunofluorescence for the presence of immune complex deposits. These examinations were performed before breaking the specimen code. Light microscopic examination was performed on 4-μm paraffin sections stained with hematoxylin and eosin.

Direct Immunofluorescence for Glomerular Immunoglobulin Deposition. Cryostat sections 5-μm thick were cut and picked up on Formolgelatin-coated slides, air-dried for 30 min, and washed twice for 15 min in phosphate-buffered saline (PBS) at pH 7.2. The slides were placed in a moist chamber, covered with antiserum, and incubated for 30 min at room temperature in the dark. Fluorescein isothiocyanate-conjugated rabbit polyvalent anti-mouse immunoglobulin (Behring Diagnostics, Sommerville, N. J.; lot 656, fluorescein:protein ratio, 2:8) was used at a titer of 1:20. Slides were rinsed quickly in PBS and washed twice for 15 min in PBS with gentle agitation, rinsed in distilled water, and mounted in glycerin-PBS. Coated sections were examined using a Wild fluorescence microscope (Wild Heerbrugg Instruments, Inc., Farmingdale, N. Y). The brightness and extent of glomerular immunoglobulin immunofluorescence was graded on a scale of 0–4 by two observers who were calibrated against each other against sections from standard specimens used in our laboratory.

Electron Microscopy. Mouse kidneys were cut immediately in cold 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3. After 1.5 h fixation at room temperature, they were fixed additionally in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.3) for 45 min. The specimens were dehydrated with alcohol and propylene oxide, infiltrated with epon-Araldite plastic, and polymerized at 75°C. Ultra-thin sections were cut on an LKB Ultratome III (LKB Instruments, Inc., Rockville, Md.), stained with uranyl acetate and lead citrate. The sections were examined by RCA-EMU (3F and 4; RCA Solid State, Somerville, N. J.) electron microscopes.

Representative areas of 2 glomeruli from each kidney were photographed and printed at a magnification ranging from 2,500 to 15,000 ×. The areas occupied by immune complexes were graded on a scale of 0–4 independently by two investigators trained in judging renal lesions as seen in electron microscopy.

Fractionation of Serum. Blood was collected from mice at monthly intervals, starting at the third postoperative month. The animals were bled from the orbital sinus. The blood was allowed to clot at room temperature for 1 h, and left at 4°C overnight. Serum was separated by centrifugation at 1,200 *g* for 10 min. Serum samples from castrated and sham-operated mice were pooled separately. 200 μl of the pooled serum samples was subjected to ultracentrifugation in a 10–35% linear sucrose density gradient (0.15 M NaCl, pH 8.0). Bovine thyroglobulin (19S), human gammaglobulin (7S), and hemoglobin (4S) were used as sedimentation markers. 40 fractions were collected and each was analyzed for antibodies to DNA and Poly A. Peak fractions were tested for immunoglobulin content. In the 19S region (fraction 10–20) only activity against μ-chain could be detected by Ouchterlony analysis, whereas in the 7S region (fractions 20–30), activity against γ-chain, but not μ-chain, was present. To determine the specificity of binding, monospecific rabbit anti-mouse μ-chain and anti-mouse γ-chain antisera were added to peak fractions from 19S and 7S regions, respectively. Anti-μ inhibited DNA binding between 77 and 100%, whereas anti-γ inhibited binding between 66 and 78%. Addition of goat anti-mouse albumin to these gradient fractions had no inhibitory effect.

Anti-DNA and Anti-Poly A Assays. Antibodies to DNA and Poly A were determined separately using a cellulose ester filter radioimmunoassay. The radioactive nucleic acids were double-stranded DNA ([^{3}H]DNA from KB cells obtained from Electro-Nucleonics Inc., Fairfield, N. J.), and [^{3}H]Polyriboadenylic acid (from Miles Laboratories Inc., Elkhart, Ind.). The radioactive antigens (750 cpm/7.74 ng Poly A; 700 cpm/3 ng DNA) were incubated with decomplemented 50-μl aliquots of each fraction for 30 min at 37°C followed by an overnight incubation at 4°C. The antigen-antibody complexes were collected onto cellulose ester filters (Millipore Corp., Bedford, Mass.). The filters were placed in counting vials and covered with 10 ml of Liquifluor-toluene scintillation medium. Radioactivity was determined in a Packard liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). The results are expressed as corrected cpm retained on the filter, a value which is directly related to serum concentration (12).

Calculation of 7S:19S Antibody Binding. The radioactive binding profiles revealed clear

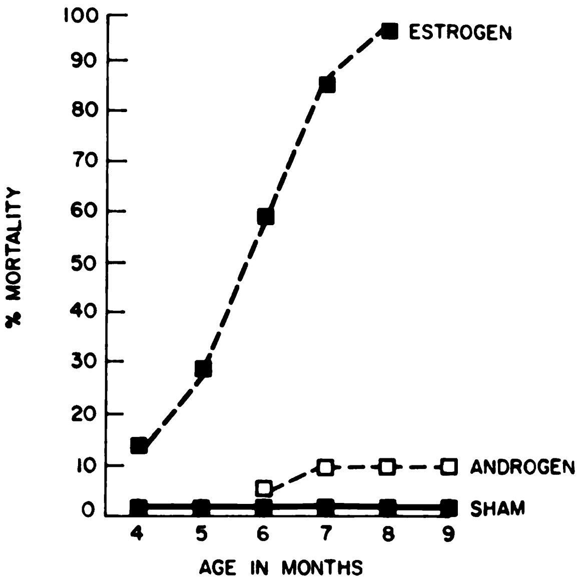

FIG. 1. Effect of prepubertal castration and sex hormone treatment on cumulative mortality in male B/W mice.

distribution into 7S and 19S peaks of activity after sucrose gradient fractionation. The radioactivity representing the fractionated 7S and 19S peaks within a single gradient were added and compared for total binding activity.

Reproducibility and Analysis of Data. To determine the reproducibility of our filter radioimmunoassay and the variations seen in age-matched mice, pooled serum samples from intact, sham-operated and variously treated male and female mice were subjected to sucrose gradient ultracentrifugation and assayed for anti-DNA antibodies. The ages ranged between 12 and 36 wk. The procedures were performed by the same technician. The radioactive DNA used was from the same lot. Some age-matched pooled samples were assayed on the same day, then repeated 1 mo later. The variations from the mean for the total anti-DNA binding in the 19S region ranged from 1.9 to 14.1%, whereas in the 7S region the range was between 0.2 and 7.1%. In the present communication, a minimum variation of 13% in the IgM and 8% in the IgG total binding activity from age- and sex-matched, sham-operated controls was required before a result was considered significant. These values represent 2 SD from the mean.

Results

Effects of Sex Hormones on Mortality of B/W Mice. Prepubertal castration combined with maintained estrogen administration caused a greatly enhanced mortality of male B/W mice (Fig. 1). 19 of 21 mice were dead by 7 mo of age. By contrast, the cumulative mortality at 1 yr was 18% for sham-operated males and 15% for androgen-treated males ($P < 0.05$). Castrated female mice given maintained estrogen also died more rapidly than sham-operated females. By contrast, castrated females given androgen had a significant decrease in mortality (Fig. 2). 8 of 14 (57%) such animals were alive at 1 yr, compared to 3 of 13 (23%) sham-operated females ($P < 0.05$). Serum dihydrotestosterone concentrations in androgen-treated male and female mice were similar to the levels of testosterone observed in sham-operated males and clearly higher than those in sham-operated females (Table I).

Effects of Sex Hormones on Antibodies to DNA and Poly A in Castrated Male

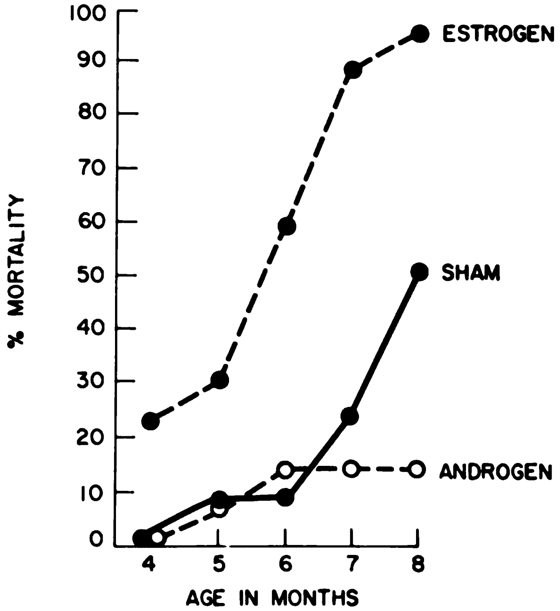

FIG. 2. Effect of prepubertal castration and sex hormone treatment on cumulative mortality in female B/W mice.

TABLE I
Serum Testosterone

Group	Age in months		
	4	5	6
		ng/ml	
Sham male	3.04	1.26	3.52
Castrated and androgen-treated male	6.50	3.33	3.10
Sham female	1.15	0.45	0.79
Castrated and androgen-treated female	3.77	3.47	3.10

B/W Mice. Castrated male mice receiving estrogen had greater DNA binding activity when compared to sham and androgen-treated male mice. The increase in IgM antibodies occurred from 3 mo onward (Fig. 3 A), whereas the major increase of IgG antibodies occurred at 6 mo (Fig. 3 B), a time of greatly accelerating mortality.

Similar results were found for antibodies to Poly A. Estrogen-treated mice had an increase in IgM from 3 mo of age (Fig. 3 C), and showed a premature appearance of IgG antibodies to Poly A at 6 mo of age (Fig. 3 D). Normally, significant amounts of IgG antibodies to Poly A do not appear in male B/W mice until 11 mo of age (6, 10).

Effects of Sex Hormones on Antibodies to DNA and Poly A in Castrated Female B/W Mice. Castrated female mice receiving androgen had less IgM and IgG binding activity for DNA when compared to sham and estrogen-treated females (Figs. 4 A and B). By contrast, mice receiving estrogen had increased DNA binding which was particularly striking at 5 mo of age. The decreased

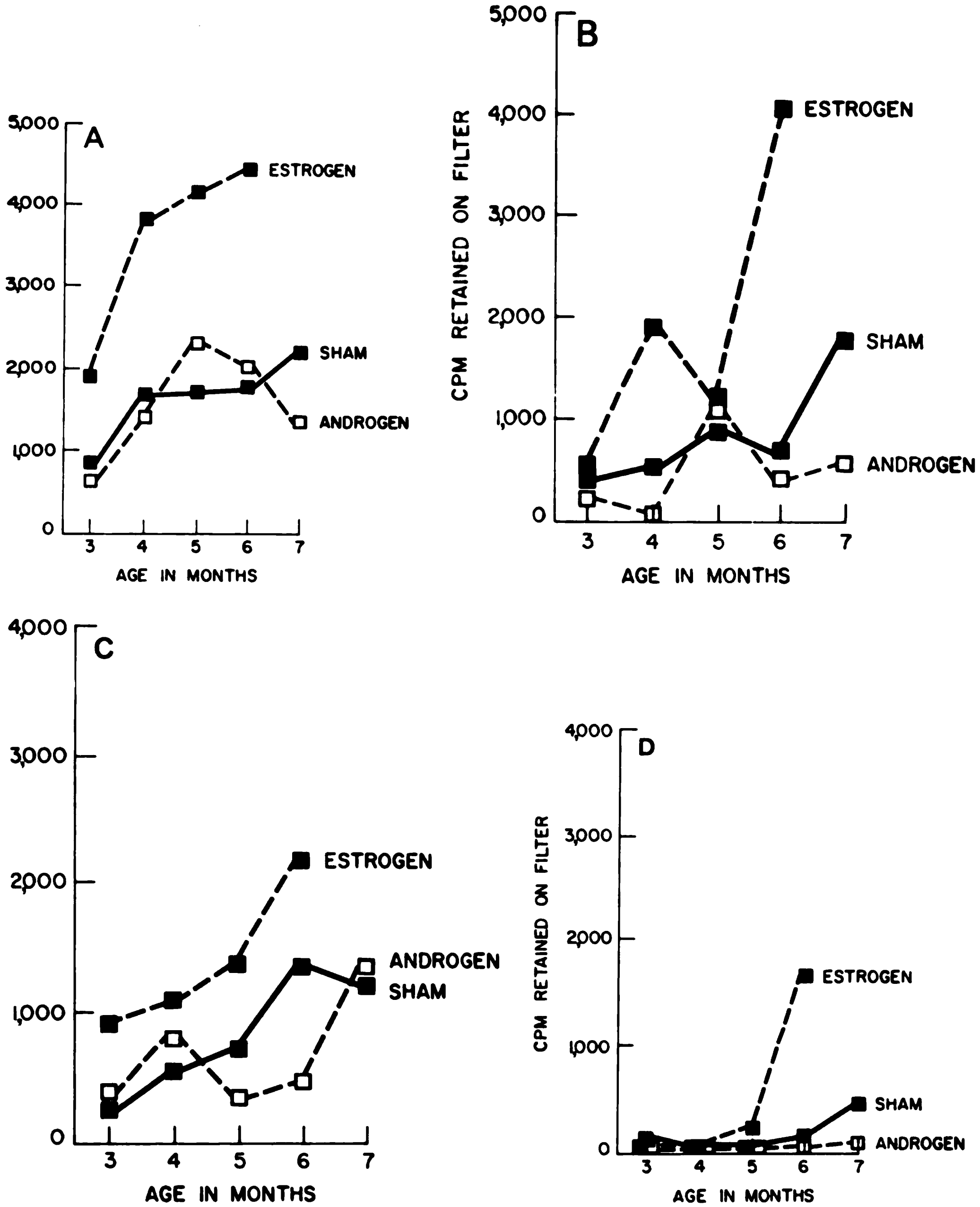

FIG. 3. Effect of prepubertal castration and sex hormone treatment of male B/W mice on (A) IgM antibodies to DNA, (B) IgG antibodies to DNA, (C) IgM antibodies to Poly A, and (D) IgG antibodies to Poly A.

binding at 6 mo may be due to the selective death of mice with the greatest amount of anti-DNA antibodies.

The effect of sex hormones on the Poly A response had the following characteristics. The augmentation due to estrogen was apparent both for IgM

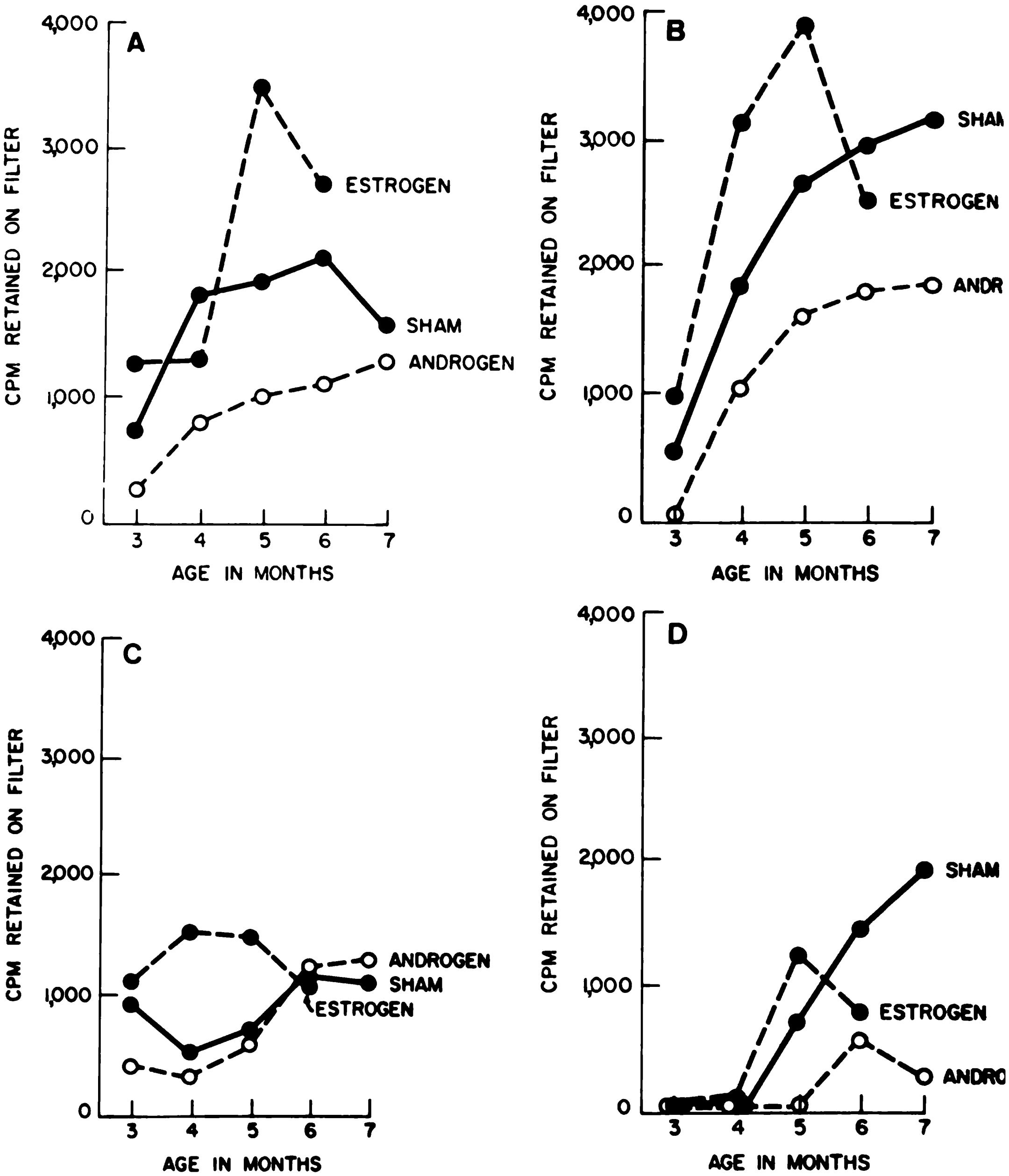

FIG. 4. Effect of prepubertal castration and sex hormone treatment of female B/W mice on (A) IgM antibodies to DNA, (B) IgG antibodies to DNA, (C) IgM antibodies to Poly A, and (D) IgG antibodies to Poly A.

(Fig. 4 C) and IgG (Fig. 4 D), and the decrease due to androgen was apparent in IgG anti-Poly A antibodies.

Effect of Sex Hormones on Renal Pathology. Sham-operated animals showed glomerulonephritis including progressive accumulation of lymphoid

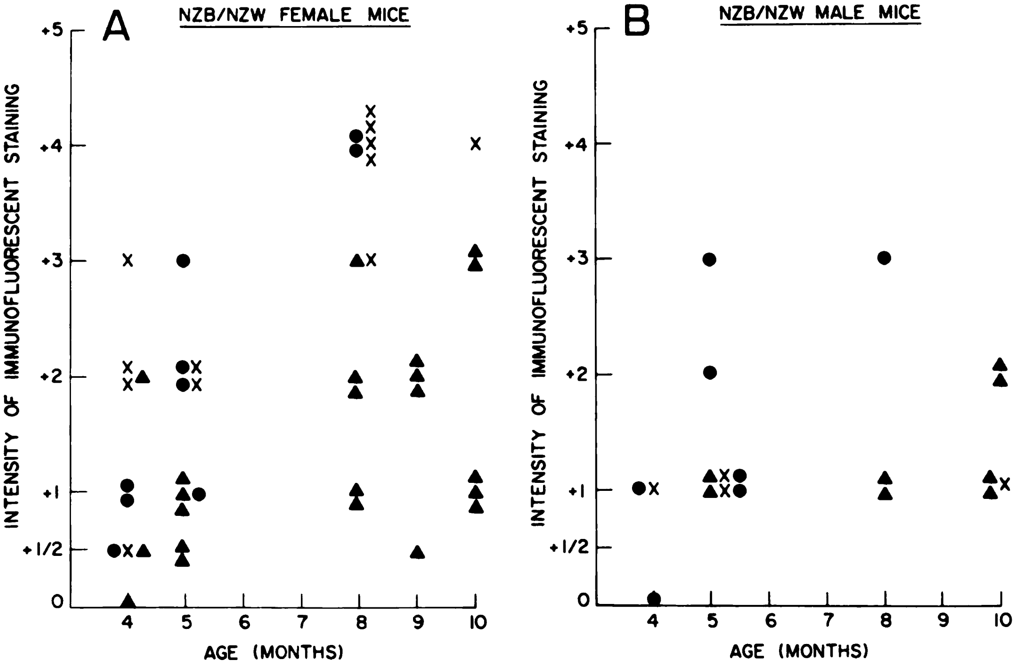

FIG. 5. Intensity of immunofluorescent staining for immunoglobulin in the glomeruli of female (A) or male (B) B/W mice at various ages. Mice were either sham-operated (X), castrated and treated with androgen (▲), or castrated and treated with estrogen (●).

cells at the calices, and subsequently around the branches of the renal artery. The glomeruli showed eosinophilic thickening of the capillary loops as early as 4 mo in sham females, with progressive glomerular enlargement and replacement by eosinophilic material. These changes were seen earlier and were more severe in sham-operated females than in sham-operated males.

At 5 and 8 mo, the lymphoid cell infiltration and glomerular abnormalities were more severe in animals which had been castrated and treated with estrogen, irrespective of sex. Furthermore, males and females castrated and given androgen showed much milder changes. This apparent exacerbation of lesions by estrogen and the converse protective effect of androgen were less apparent at very early and very late stages.

Immunofluorescence. Glomeruli showed significant granular staining for immunoglobulin at the earliest age examined (4 mo). Sham-operated females showed more extensive and intense immunofluorescence than sham-operated males. The exacerbation of the glomerular changes by estrogen and the protective effects of androgen were apparent in both sexes, particularly at 5 and 8 mo (Figs. 5 and 6). At 9 and 10 mo, many castrated females treated with androgen were still alive and had relatively fewer glomerular deposits of immunoglobulin compared to the few remaining sham females.

Electron Microscopy. The electron microscopic abnormalities present in the kidneys of B/W mice have been well described (13). In our studies, electron microscopic observation confirmed the renal involvement suggested by the mortality and immunopathologic findings discussed above.

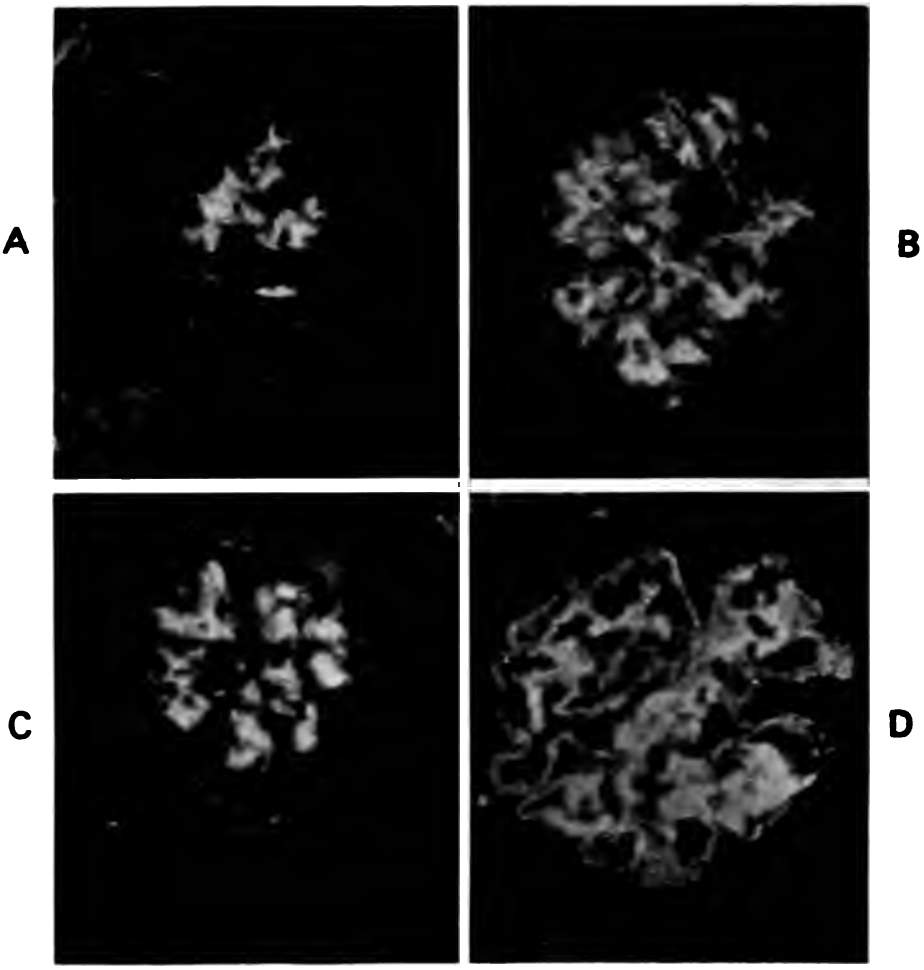

FIG. 6. Representative examples of immunofluorescent analysis of kidneys from castrated, 8-mo-old B/W mice: (A) B/W male, castrated and treated with androgen (1 plus); (B) B/W male, castrated and treated with estrogen (3 plus); (C) B/W female, castrated and treated with androgen (2 plus); and (D) B/W female, castrated and treated with estrogen (4 plus). Original magnification ×312.

Sham-operated females at 4 mo already showed electron dense deposits in their glomeruli. Renal deposits were apparent at 5 mo in the castrated and estrogen-implanted mice, both female and male. These mice showed deposits present in the mesangial area, while castrated and androgen-implanted males at 5 mo had no deposits. At 10 mo, androgen-implanted animals still showed some protection from renal involvement (Fig. 7 A) when compared to a sham-operated female (Fig. 7 B).

Retreatment with Androgen at 9 Mo of Age. By 8 mo of age, antibodies to Poly A were essentially the same in sham-operated animals and in those that had received androgen once prepubertally (Table II). Without further androgen, IgG antibodies to Poly A showed the expected rise at 10 and 11 mo of age in both

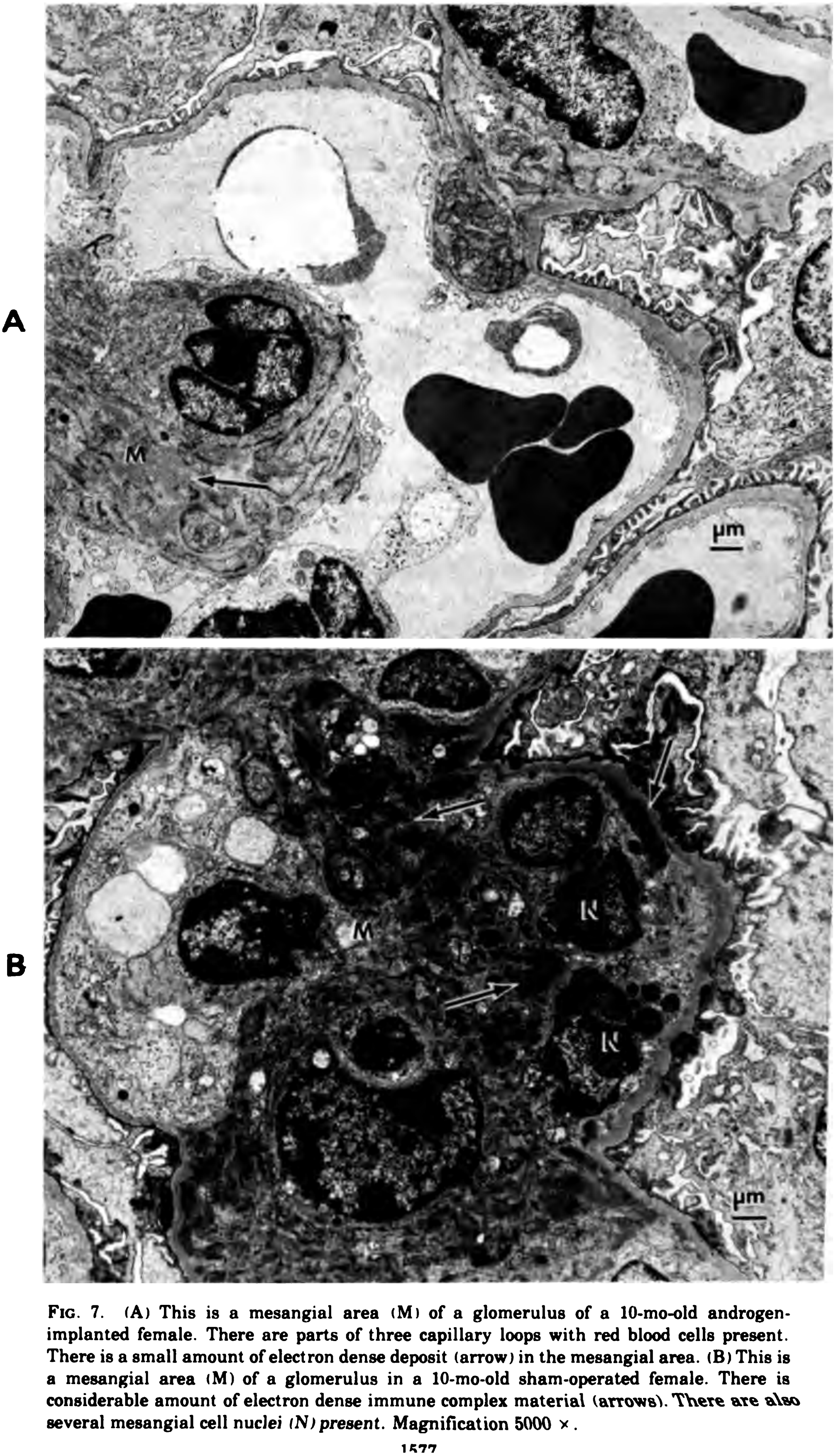

FIG. 7. (A) This is a mesangial area (M) of a glomerulus of a 10-mo-old androgen-implanted female. There are parts of three capillary loops with red blood cells present. There is a small amount of electron dense deposit (arrow) in the mesangial area. (B) This is a mesangial area (M) of a glomerulus in a 10-mo-old sham-operated female. There is considerable amount of electron dense immune complex material (arrows). There are also several mesangial cell nuclei (N) present. Magnification 5000 ×.

TABLE II

Effect of a Second Exposure to Maintained Androgen Treatment at 9 Mo of Age

Group	Antibodies to Poly A (ng Poly A bound/0.2 ml serum)							
	8 Mo		9 Mo		10 Mo		11 Mo	
	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
Male								
Sham	96.6	24.6	120.1	20.5	127.8	159.8	76	92.5
Androgen once	120	27.6	131.2	21.6	130.1	130	95	87.4
Androgen twice					101.9*	49.1*	92	12.8*
Female								
Sham	88.9	159.4	99.1	204.3	116.5	497	90.4	375.8
Androgen once	97.8	182.3	—	—	—	—	—	—
Androgen twice	—	—	86.3	103.7*	58.9*	117.9*	48.9*	63.5*

* A value greater than 2 SD from the mean.

sexes. This suggested that the suppressive effect of androgen had worn off by this time, and led us to attempt a second reimplantation of an androgen-containing capsule. The introduction of this additional male hormone at 9 mo of age aborted the development of IgG antibodies to Poly A in both male and female castrated mice (Table II).

Androgen Treatment of Intact Males at 8 Mo of Age. We have observed a spontaneous fall in serum testosterone concentration occurring in noncastrated 9-mo-old male B/W mice (from 4.7 ng/ml to 1.0 ng/ml). After 9 mo, these males develop an accelerated disease associated with high concentrations of IgG antibodies to DNA (6, 10). This temporal correlation between the decline in androgen concentration and worsening of disease led us to institute androgen treatment in intact males at 8 mo of age by the implantation of hormone-containing capsules.

The treated male B/W mice had significantly fewer IgG antibodies to DNA and Poly A when compared to the sham-treated animals. Moreover, the expected switch to IgG antibodies to Poly A was not seen in the treated mice (Figs. 8 A and B). At 19 mo of age, 10 of 14 (71%) treated mice were alive, compared to 4 of 9 (44%) sham-implanted controls ($P < 0.005$).

Combined Neonatal Thymectomy and Perinatal Castration of Male B/W Mice. We have previously reported that neonatal thymectomy of male B/W mice results in an accelerated disease associated with premature development of IgG antibodies to DNA and enhanced mortality (10). By contrast, neonatal thymectomy prevents the development of IgG antibedies to Poly A, suggesting that this IgM to IgG switch is thymic-dependent (Fig. 9).

Perinatal castration of male B/W mice also decreases survival and leads to early development of IgG antibodies to DNA (8). In these respects, castration resembles neonatal thymectomy. However, whereas the development of IgG antibodies to Poly A is virtually abolished by thymectomy (10), it is promoted by castration of males (ref. 8, Fig. 9). These contrasting effects on the Poly A response offered an opportunity to study whether or not this effect of castration required the thymus.

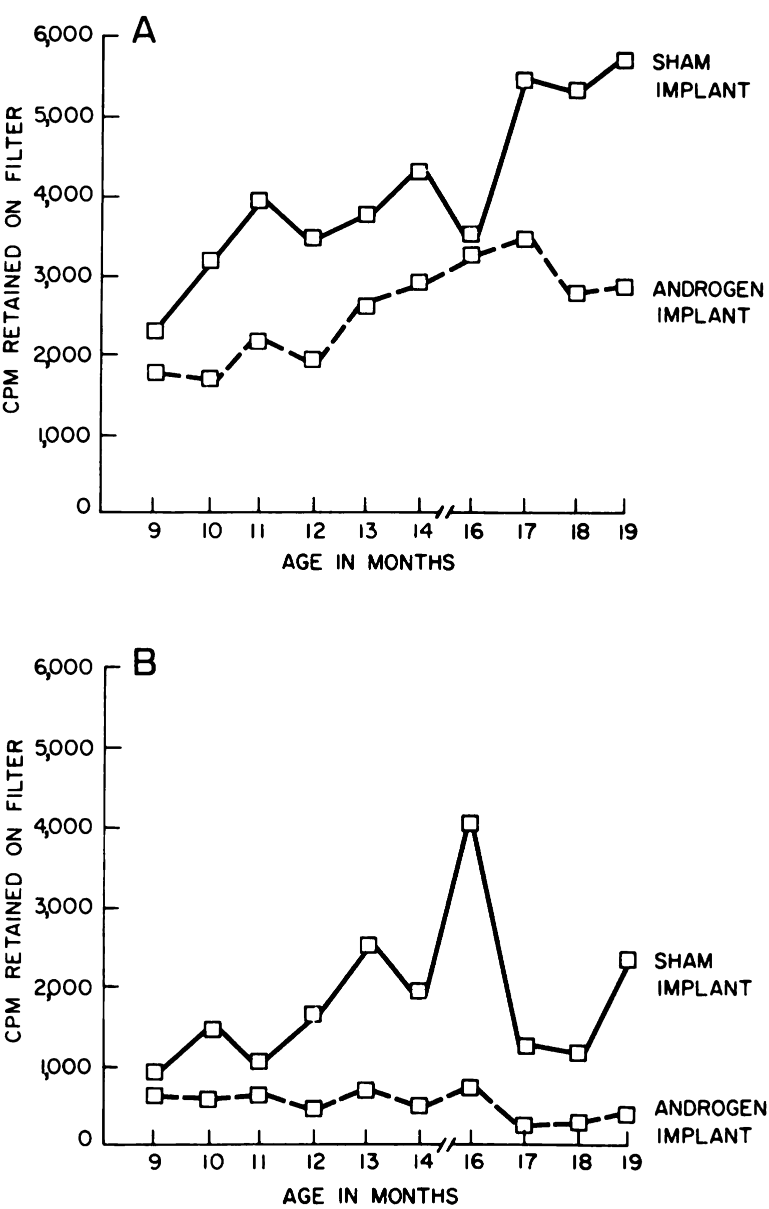

FIG. 8. Effect of androgen treatment of intact, 8-mo-old male B/W mice on (A) IgG antibodies to DNA and (B) IgG antibodies to Poly A.

Male B/W mice subjected to combined neonatal thymectomy and perinatal castration developed the expected accelerated disease but failed to develop IgG antibodies to Poly A despite the castration (Fig. 9). Therefore, the premature appearance of IgG antibodies to Poly A that usually follows castration was prevented if thymectomy was also performed. Thus, the dominant influence on the IgG Poly A response was attributable to the thymus rather than the gonads.

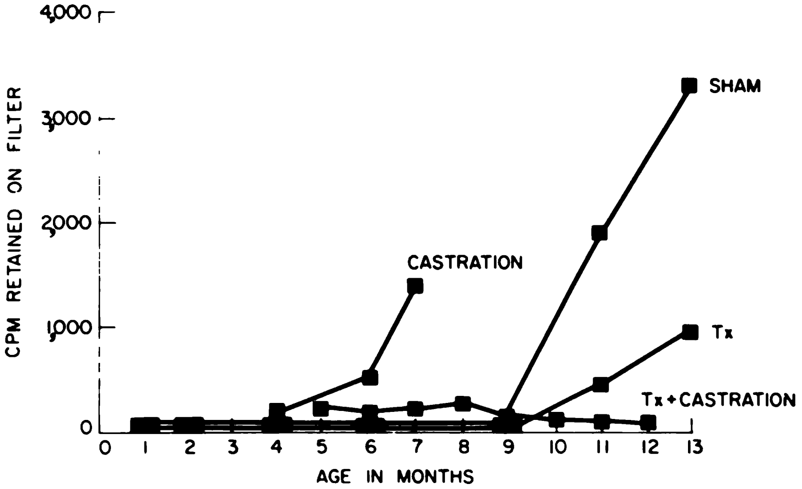

FIG. 9. Effect of prepubertal castration, neonatal thymectomy (Tx) and combined neonatal thymectomy and prepubertal castration on IgG antibodies to Poly A in male B/W mice.

Discussion

This study demonstrates that both androgens and estrogens can modify the expression of autoimmunity in B/W mice, with male hormone suppressing and female hormone accelerating disease. The sex hormones influence survival, the formation of antibodies to DNA and Poly A, and the severity of immune complex nephritis. These results extend earlier reports that prepubertal castration alone caused a more severe disease in male B/W mice, suggesting a suppressive effect of androgen (8, 14). The combination of castration plus maintained hormone administration used in the current study showed an effect of female hormone as well.

The immunofluorescent and electron microscopic findings suggest that the effect of these hormones on antibodies to nucleic acids and on survival are mirrored in the renal histopathology. Gamma globulin deposition and electron dense deposits are reduced in androgen-treated mice, and increased in estrogen-treated animals. The mechanisms accountable for these findings might involve antibody formation, complement activation, inflammatory cell recruitment, or any combination of these or other factors. Future experiments may be able to define these mechanisms more precisely. It is of interest that certain complement components are dependent on androgen for their expression (15).

These effects of sex hormones probably explain the greater incidence of lupus and other autoimmune disorders in females (16), and the tendency to autoimmunity seen in Klinefelter's syndrome (17). It is interesting that the female:male ratio in lupus is greatest between menarche and menopause, and declines before puberty and with old age (16). An abnormality in estrogen metabolism perhaps resulting in an increased estrogen activity has recently been demonstrated in Klinefelter's syndrome (18). Moreover, it has been shown that the testes in these patients may secrete 10 times the normal amount of estrogen (19).

These opposing effects of sex hormones are not unique to B/W mice, since female mice of normal strains have greater humoral and cellular immune responses than their male littermates (20, 21). Androgens tend to cause lymphocyte depletion (22, 23) and decreased immunoglobulin concentrations (24). Orchidectomy causes an increase in immunologic reactivity (25). There is some suggestion that the suppressing effects of androgen require the presence of the thymus (21). Our studies in mice subjected to combined castration and neonatal thymectomy support this hypothesis. A simple notion consistent with current concepts of immunologic regulation would be that androgens promote the function of suppressor T cells, whereas estrogens favor the development of helper T cells.

The two experiments in which androgen treatment is instituted between 8 and 9 mo of age (either by reimplantation or treatment of intact males) suggest that male hormone may exert a suppressor effect even in older animals. Intact males treated in this manner live significantly longer than sham-implanted controls. These results suggest that androgen treatment may be beneficial in mice with already established autoimmune disease. We have also observed that female B/W mice treated with androgen starting at 3 or 6 mo of age live longer than sham-treated controls.[2] The therapeutic possibilities inherent in androgen administration may be worth consideration in human lupus.

Summary

NZB/NZW F_1 mice of both sexes were castrated at 2 wk of age and implanted subcutaneously with silastic tubes containing either 5-α-dihydrotestosterone or estradiol-17-β. Mice receiving androgen showed improved survival, reduced anti-nucleic acid antibodies, or less evidence of glomerulonephritis as determined by light, immunofluorescent, and electron microscopy. By contrast, opposite effects were observed in castrated mice receiving estrogen.

Intact male NZB/NZW F_1 mice received androgen implants at 8 mo, an age when they develop an accelerated autoimmune disease associated with a decline in serum testosterone concentration. Such treated mice had improved survival and reduced concentrations of antibodies to DNA and to polyadenylic acid (Poly A).

Prepubertal castration of male NZB/NZW F_1 mice results in an earlier appearance of IgG antibodies to Poly A. This effect of castration was prevented if neonatal thymectomy was also performed.

We gratefully acknowledge the technical assistance of Ms. Jackie Sudakian, Ms. Carmen Guillen, and Mr. Robert Surface, and the help of Ms. Carol Evans-Foster in preparation of the manuscript.

Received for publication 18 November 1978.

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AFFINITY OF FIBRONECTIN TO COLLAGENS OF DIFFERENT GENETIC TYPES AND TO FIBRINOGEN*

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Fibronectin is a major cell surface protein of normal fibroblasts (1–4) but is absent from the surface of transformed fibroblasts (2, 5–8). Fibronectin is also present in plasma (1, 9). It has affinity to fibrinogen and fibrin and is identical with cold insoluble globulin (10–12). A recent comparison of fibronectins derived from human plasma and fibroblasts by analysis of polypeptide chains, peptide maps, and amino acid and carbohydrate compositions, strongly suggests that they are the same protein (13). Fibronectin is present in the fibroblasts of different species as immunologically cross-reactive but not identical components (14). Recent results from our laboratory show that human fibronectin, whether derived from cultured fibroblasts or plasma, binds to collagen (15).

During the past several years it has become clear that collagen exists as several distinct types determined by independent structural genes (16, 17). Type I collagen is the most prevalent form of collagen in the mature vertebrate organism and occurs in the arterial walls, bone, dentin, dermis, tendon, and uterine wall. Type II is almost exclusively limited to hyaline cartilages. Type III collagen has a distribution pattern similar to that of type I, but is more prevalent in the distensible soft connective tissue. Recently, additional collagen chains, provisionally designated as the A chain and B chain, have been isolated from extracts of several tissues (18, 19), and it has been suggested that molecules comprising these chains may originate in certain basement membrane structures.

In view of the possible importance of the fibronectin-collagen interaction to the structure of the various connective tissues, we decided to investigate the affinity of fibronectin to the various genetic types of collagen. We show here that all types of collagen tested are active in fibronectin binding, but to a different degree. Further, we demonstrate that the collagen binding activity is shared by fibronectins of a wide variety of species. We also present evidence that the binding to collagen and fibrinogen is mediated by the same binding site in fibronectin.

Materials and Methods

Blood was collected in sodium citrate, and plasma was separated by centrifugation at room temperature. Fresh plasma was used for the purification, or when this was not possible, the plasma was stored frozen at −20°C. Chicken plasma was obtained from Pel-Freez Farms, Inc.,

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* Supported by grants CA 22108 and CA 16434 from the National Cancer Institute, Department of Health, Education, and Welfare.

(Rogers, Ark.) and plasma from *Torpedo californica* from Pacific Bio-Marine Laboratories, Inc. (Venice, Calif.).

Confluent cultures of chicken fibroblasts were kindly provided by Dr. J. Strauss, California Institute of Technology. The cultures were washed three times with phosphate-buffered saline (PBS)[1] and extracted with 8 M urea, 0.05 M Tris-HCl, pH 7.0, containing 10^{-4} M phenylmethanesulphonyl fluoride (PMSF). Five roller bottles were extracted twice with a total of 100 ml of the urea solution. The extracts were combined, centrifuged, and stored frozen. Gelatin (swine skin, 300 Bloom), poly-L-proline (mol wt 10,000–30,000), and cyanogen bromide activated Sepharose were from Sigma Chemical Co. (St. Louis, Mo.), and keratin from ICN, K & K Laboratories, Inc., (Plainview, N. Y.). Pig tropoelastin was a gift from Dr. W. Gray, University of Utah, and a linear polymer of the tripeptide pro-gly-pro was provided by Dr. S. Fuchs, the Weizmann Institute (Rehovot, Israel). Fibrinogen was obtained from Kabi AB (Stockholm, Sweden) and was purified further to remove contaminating fibronectin as described (10).

Purification of Fibronectin. Plasma fibronectin was purified essentially as described earlier (15) using gelatin insolubilized on Sepharose. The modifications were that the plasma sample was passed through a column of plain Sepharose 4B before fractionation on gelatin-Sepharose to remove material binding to Sepharose. The amount of Sepharose was twice the volume of the plasma sample. The gelatin-Sepharose column was washed with PBS containing 10^{-4} PMSF and 1 M urea in 0.05 M Tris buffer, pH 7.5, and the fibronectin was eluted with 4 M urea in 0.05 M Tris buffer. Urea extracts of fibroblasts were diluted 20 times with PBS, and 1 ml of gelatin-Sepharose was added per 200 ml of solution. After incubation for 16 h at 4°C, the particles were packed in a column, washed, and eluted as described above for plasma fibronectin.

Purification of the Collagens. Type I and III collagens were prepared from human placenta after solubilization by limited digestion with pepsin and fractionation by differential salt precipitation at neutral pH as described previously (20). Briefly, these collagens were precipitated from the initial pepsin digest by adding NaCl to a concentration of 0.7 M. The precipitate was collected, redissolved in 1.0 M NaCl, pH 7.5 (0.05 M Tris), and type III collagen was selectively precipitated by increasing the NaCl concentration to 1.5 M. Type I collagen was similarly precipitated at 2.5 M NaCl. After the initial precipitation of type III and I collagens at 1.5 and 2.5 M NaCl, respectively, the precipitation from neutral salt solutions was repeated three times for each collagen.

Collagen molecules comprising the A and B chains were isolated from the supernatant solution of the original pepsin digest after precipitation of the type I and III collagens (18). For this purpose, the NaCl concentration was raised from 0.7 to 1.2 M. The resulting precipitate was collected and redissolved in 1.0 M NaCl, pH 7.5 (0.05 M Tris). The collagen was then selectively precipitated from the latter solution at 4.5 M NaCl (19). The precipitation from the neutral salt solution was repeated three times.

Type II collagen was prepared from infant articular cartilage. This collagen was likewise solubilized by limited digestion with pepsin and purified as previously described (21). The solubilized collagen was precipitated from the pepsin digest by the addition of NaCl to a concentration of 0.9 M. The precipitate was redissolved in 1.0 M NaCl, pH 7.5 (0.05 M Tris), and the collagen was selectively precipitated from this solution by increasing the NaCl concentration to 3.2 M. The latter precipitation was repeated three times.

All of the collagens purified for the present study were ultimately redissolved in 0.5 M acetic acid, dialyzed extensively against the solvent, and lyophilized.

The cyanogen bromide peptides, $\alpha 1$(II)-CB8 and $\alpha 1$(II)-CB12, were isolated from the cleavage products of human $\alpha 1$(II) chains as previously described (22).

Before testing, collagens and peptides were dissolved in 0.2% acetic acid. Denaturation of collagens was obtained by warming to 60°C for 30 min followed by rapid cooling in ice.

Antisera. Antisera were prepared against human and chicken plasma fibronectins. The latter were purified by the gelatin-Sepharose method described above. The antisera were adsorbed with Sepharose-conjugated plasma proteins obtained from plasma passed through gelatin-Sepharose

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[1] *Abbreviations used in this paper:* CAF, cell attachment factor; CAP, cell attachment protein; ELISA, enzyme-linked immunosorbent assays; PBS, phosphate-buffered saline; PMSF, phenylmethanesulphonyl fluoride; SDS, sodium dodecyl sulphate.

(and devoid of fibronectin), bovine plasma proteins (including fibronectin) conjugated to Sepharose, and with gelatin-Sepharose. Antibodies to fibronectin were isolated from the absorbed antisera by absorption to Sepharose-conjugated fibronectin followed by elution with 8 M urea.

Assays for Fibronectin and Collagen Binding. Quantitation of fibronectin was performed by radioimmunoassay as detailed elsewhere (23). Tests for binding and inhibition of binding of fibronectin to various substances were performed using enzyme-linked immunosorbent assays (ELISA) (15, 24) performed in polystyrene microtiter plates (ELISA plates, Cooke Laboratory Products Div., Dynatech Laboratories Inc., Alexandria, Va.). For binding assays, the wells were coated by incubation at room temperature with solutions containing 1 μg/ml of various proteins. Purified fibronectin was incubated in washed, coated wells for 6 h. After washing of the plate, bound fibronectin was quantitated by incubation overnight with excess enzyme-labeled, purified antibodies to fibronectin.

For inhibition assays, the microtiter plate wells were coated with 0.5 μg/ml of gelatin. Human or chicken fibronectin, 0.5 μg/ml, with or without various concentrations of inhibitor was incubated in the wells, and the amount of fibronectin bound to the wells was detected with enzyme conjugated antibodies as described above. All incubations were performed at 4°C. Enzyme activities were measured at room temperature.

Gel electrophoresis was performed in cylindrical (25) gels in the presence of sodium dodecyl sulphate (SDS). Protein was determined according to Lowry et al. (26).

Results

Purification of Fibronectins. We have previously shown that fibronectin from human plasma and spent culture media of human fibroblasts can be isolated on gelatin-Sepharose (15). Similar binding to gelatin-Sepharose was found with fibronectins from chicken plasma and fibroblast extracts. Both preparations showed one major band in electrophoresis (Fig. 1). A slight difference was repeatedly found in the mobilities of the fibronectins from the two sources. The fibroblast fibronectin was slightly slower. A protein with a mobility similar to that of mammalian and chicken fibronectins in SDS gel electrophoresis was obtained from plasma of Torpedo fish (*Torpedo californica*). When run in SDS gel electrophoresis in the absence of reducing agent, it also behaved like fibronectin, giving a band with an apparent molecular weight of about twice the size of the subunit. The yield of this protein, tentatively identified as fibronectin, was comparable to that obtained with mammalian fibronectins.

Binding of Fibronectin to Collagen, Fibrinogen, and Other Proteins and Glycoproteins. Our recent results have established that fibronectin exhibits an affinity for collagen and gelatin (15). The binding of fibronectin to collagen was further characterized using different types of human collagen. It has also been shown that fibronectin has affinity to fibrinogen and fibrin (10). Since fibrinogen and collagen do not have much in common, it was important to compare these binding activities and to test other proteins for binding of fibronectin.

For assays of binding of fibronectin, microtiter plates were coated with various proteins. All four preparations of collagen were found to bind fibronectin. The denatured forms of the collagens had a higher binding capacity than the native collagens. Of the latter, type III collagen was the most efficient (Fig. 2). Fibrinogen bound much less fibronectin than the collagens, and other proteins tested did not show significant binding in this assay. The binding of fibronectin to fibrinogen as well as to collagens was completely inhibited by gelatin, but not with ovalbumin used as a control. Furthermore, the binding of

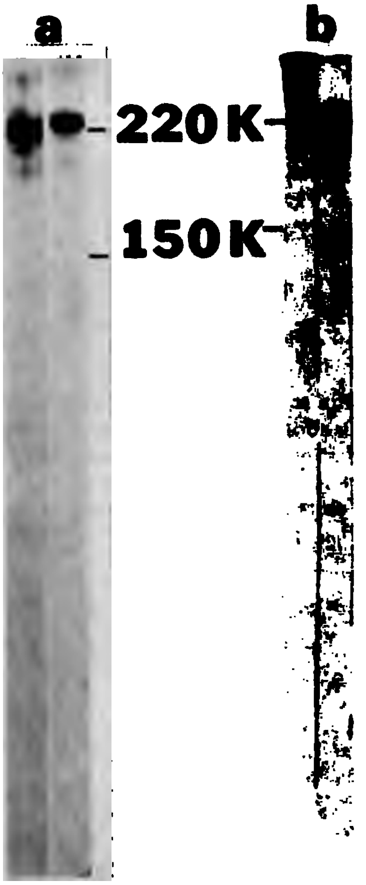

FIG. 1. Gel electrophoresis in the presence of SDS using cylindrical gels with 5% polyacrylamide. a. Chicken plasma fibronectin (left) and chicken fibroblast fibronectin (right). Samples were reduced with 2% 2-mercaptoethanol. b. Fibronectin from *Torpedo californica* plasma. The sample in the left gel was run without reduction, and that in the right gel was reduced. The positions of unreduced IgG (mol wt 150,000) and reduced human plasma fibronectin (mol wt 220,000) are shown.

fibronectin to fibrinogen was inhibited by lower concentrations of gelatin than of fibrinogen itself (not shown).

Lack of Self-Aggregation of Fibronectin. We have noticed that purified fibronectin has a tendency to aggregate in concentrated solutions. Preparations of purified fibronectin eluted from the gelatin-Sepharose columns in concentrations of 1–3 mg/ml have often formed a gel-like precipitate upon freezing and storing, even in the presence of 8 M urea. This prompted us to examine fibronectin for self-associating properties. Fibronectin was coupled to Sepharose, and the binding of ^{125}I-labeled fibronectin to the particles was studied. The radioactivity bound to fibronectin-Sepharose was the same (6%) as binding to particles to which albumin was coupled, while gelatin-Sepharose bound more than 90% of the labeled protein.

Avidity of Binding of Fibronectin to Collagens and Fibrinogen. The binding

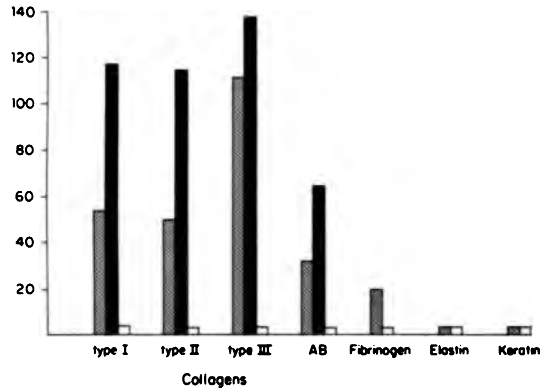

FIG. 2. Binding of fibronectin to microtiter plate wells coated with different proteins as detected by enzyme labeled antifibronectin. Results are expressed as relative enzyme activities (%) compared to that obtained when the wells were coated with gelatin (100%). Hatched bars, binding to wells coated with native proteins, solid bars, binding to wells coated with heat denatured proteins, open bars, binding in presence of 100 μg/ml gelatin.

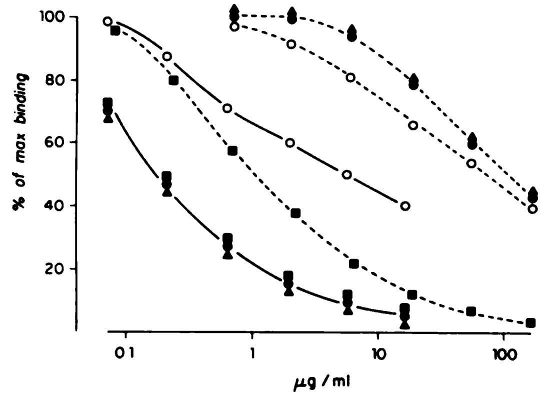

FIG. 3. Inhibition of binding of human fibronectin to microtiter plates coated with gelatin by collagen type I (●), type II (▲), type III (■), and AB chains (O). Hatched lines, native proteins. Solid lines, heat denatured proteins.

of fibronectin to the different collagens was further characterized by testing the capacity of the collagens to inhibit binding of fibronectin to gelatin. Of the native collagens, type III was the most efficient inhibitor (Fig. 3). Heat denaturation of the collagens abolished the differences between the activity of type I, II, and III collagens, all of which became much more efficient inhibitors. Denaturation had less effect on molecules composed of A and B chains, which remained less active than denatured type I, II, and III collagens.

Chicken fibronectin showed a similar preference for denatured collagens and native type III collagen. A slight difference between the assay patterns obtained with chicken and human fibronectins were found, but chicken fibronectins derived from plasma, and fibroblasts, respectively, showed identical specificities (Table I).

Human fibrinogen had some capacity to inhibit fibronectin-gelatin interac-

TABLE I

Inhibition of Binding of Chicken Fibronectin, Isolated from Plasma or Fibroblasts, to Gelatin-Coated Microtiter Plate Wells by Human Collagens

	μg/ml of collagen required for 50% inhibition of	
	Plasma fibronectin	Fibroblast fibronectin
Native collagen type		
I	300	280
II	>100	>100
III	7.5	7.5
AB	>200	>200
Denatured collagen type		
I	1.5	1.7
II	0.8	1.1
III	1.3	1.6
AB	130	150

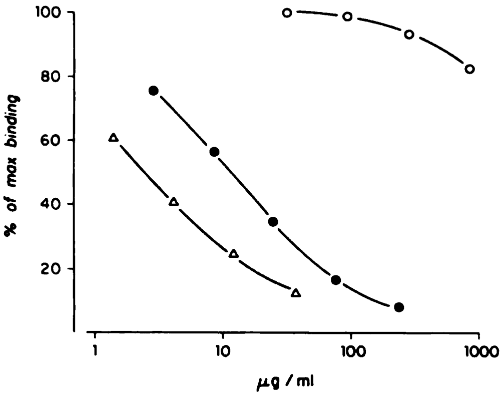

FIG. 4. Inhibition of binding of human fibronectin to gelatin coated plates by fibrinogen (○), *Torpedo californica* fibronectin (●), and rabbit fibronectin (△).

tion at high concentrations (Fig. 4). Serum albumin, ovalbumin, transferrin, and IgG did not inhibit when similarly tested. Fig. 4 also shows a comparison of the gelatin-binding activity of Torpedo fibronectin with rabbit fibronectin. Torpedo fibronectin competed efficiently with human fibronectin for binding to gelatin but was somewhat less active than rabbit fibronectin. The latter was used for comparison because rabbit antibodies to human fibronectin were used to detect the binding, and such antibodies do not react with rabbit (or Torpedo) fibronectin (14, 27). Torpedo fibronectin was also able to inhibit the binding of chicken fibronectin to gelatin and was in this assay more efficient than a preparation of bovine fibronectin (not shown).

Localization of Fibronectin Binding in Collagen Polypeptide Chains. To study the question whether fibronectin binds to a single site or multiple sites on

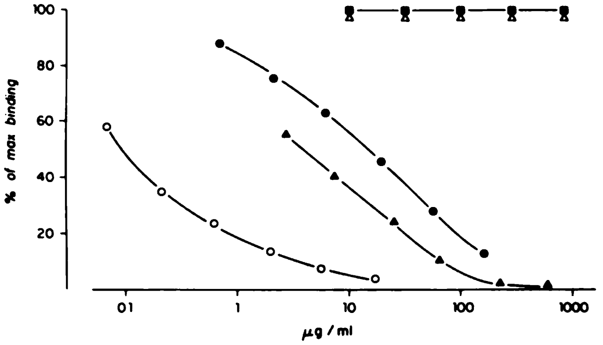

FIG. 5. Inhibition of binding of human fibronectin to gelatin-coated microtiter plate wells by denatured type II collagen (○) and two cyanogen bromide fragments of α1(II) chains, CB8 (●), and CB12 (▲). The synthetic polymer of pro-gly-pro (△), as well as polyproline (■), do not inhibit.

TABLE II

Binding of Fibronectin to Fibrin Clot in the Presence and Absence of Gelatin

	Sample	Temperature	Gelatin	Fibronectin	
				μg/ml	%
Exp. 1	Plasma	+4°C	–	270	100
	Serum	+4°C	–	10	4
	Serum	+4°C	2 mg/ml	110	40
	Serum	+22°C	–	115	42
	Serum	+22°C	2 mg/ml	205	75
Exp. 2	Plasma	+4°C	–	330	100
	Serum	+4°C	–	17	5
	Serum	+4°C	2 mg/ml	218	66
	Serum	+37°C	–	172	52
	Serum	+37°C	2 mg/ml	135	41

the collagen chains, cyanogen bromide peptides from different parts of the α1(II) chain were analyzed in the inhibition assay. Peptides CB8 (13,000 daltons) and CB12 (8,000 daltons) were both able to inhibit completely the binding of fibronectin to gelatin (Fig. 5). Both peptides were more active than native type II collagen but less active than denatured type II collagen, which yields intact α1(II) chains. The smaller of the two peptides, CB12, was more active than the larger, CB8. A polymer of a synthetic tripeptide pro-gly-pro, which has a secondary structure similar to collagen and has been shown to elicit an immune response against collagen (28), did not inhibit the fibronectin-gelatin interaction in concentrations up to 1 mg/ml. Polyproline was also inactive.

Effect of Gelatin on Incorporation of Fibronectin in Blood Clot. Clotting of blood in the cold results in binding of fibronectin to the fibrin clot (10). The addition of gelatin to blood before coagulation partially prevented the incorporation of fibronectin into the blood clot (Table II). Gelatin did not prevent or slow down coagulation, but rather encouraged it, and no fibrinogen could be detected in serum from gelatin containing plasma as tested by immunodiffusion against antifibrinogen serum.

Discussion

We have previously demonstrated an affinity of the soluble form of human fibronectin from fibroblasts and plasma for collagen and gelatin (15). The present results extend this to chicken fibronectin. A protein of Torpedo fish plasma with molecular properties similar to the mammalian and chicken fibronectins could also be isolated by utilizing the same affinity chromatography procedure that results in isolation of mammalian and chicken fibronectins. The fact that the fish protein could be purified on gelatin-Sepharose implies an affinity for collagen. Moreover, the fish protein competed with human and chicken fibronectins for binding to collagen indicating a similar specificity. Furthermore, it behaved similarly to fibronectin in SDS gel electrophoresis. This evidence allows the tentative conclusion that this protein is a homologue of the mammalian and chicken fibronectins. This, and the fact that we have been able to detect and isolate fibronectin from the plasma of every mammalian species studied (E. Engvall, and E. Ruoslahti, unpublished results), shows that the affinity of fibronectin to collagen is shared by a variety of species ranging from human to fish.

Our results show that fibronectin is the only major component from plasma that binds to gelatin-Sepharose. It also is the only major component of fibroblast extracts that shows this property, as demonstrated by the purification of homogeneous fibronectin from extracts of chicken fibroblasts. These results strongly suggest that the fibronectin-collagen interaction is not fortuitous, but, instead, is likely to have biological significance.

Since fibronectin is a cell surface component, it is possible that fibronectin could attach cells to the extracellular matrix. There is direct evidence from in vitro experiments that this is the case. A plasma component known as cell attachment protein (CAP) and a cell surface component called cell attachment factor (CAF) mediate the attachment of cultured fibroblasts to collagen coated plates (29, 30). CAF and CAP are apparently identical to fibronectin (30, 31). To gain insight into what collagen-fibronectin interactions might occur in vivo, we analyzed the relative capacities of different genetic types of collagens and their unfolded derivatives to bind fibronectin. It was found that fibronectin showed preference to the denatured forms of the collagens. A similar preference is shown by some of the enzymes active in the post-translational modifications of collagen, e.g., the glucosyl and galactosyl transferases as well as the prolyl and lysyl hydroxylases (32). It is unlikely that fibronectin would be identical to any of these enzymes, because they are mainly intracellular and present in lower amounts than fibronectin.

Limited information is available on the nature of the fibronectin binding site in collagen. The fact that commercial gelatin preparations and heat-denatured collagens are active indicates that the binding site does not depend on an intact helical structure of collagen.

We have also found that two peptides representing different parts of the $\alpha 1(\mathrm{II})$ chain are both able to inhibit completely the binding of fibronectin to gelatin. The CAP-mediated attachment of cells to collagen-coated plates has also been shown to be inhibited by several different cyanogen bromide fragments of the $\alpha 1(I)$, $\alpha 2$, and $\alpha 1(II)$ chains (33). However, the specific peptides we have tested were not highly active in the cell attachment test. This is probably due to lower sensitivity of the latter test as compared to our assay. These results, therefore, permit the tentative conclusion that the binding sites occur at several locations in the collagen sequence. However, it has been shown earlier (15) that free proline, hydroxyproline, lysine, glycine, glucose, and galactose do not inhibit the fibronectin-collagen interactions. Moreover, the present results indicate that the linear polymers [proline-glycine-proline] and polyproline are also inactive.

Immunofluorescence shows that fibronectin is especially abundant in the basement membranes (34). It was somewhat surprising that the A and B chains, which are thought to be derived from certain basement membranes, are only moderately active in fibronectin binding. Accordingly, it will be of great interest to test other collagens derived from basement membranes for fibronectin binding capacity. Since the specialized functions of the different collagens are not completely known, it is difficult to assess the significance of the differences in the activity of the different collagens in fibronectin binding. This may have little influence in vivo. Since all collagens are active to some extent, it may be the types and relative amounts of collagen available at a given location, rather than the relative binding activities, that determines which collagen interacts with fibronectin.

Fibronectin also binds to fibrinogen and fibrin (10, 12). Our results show that this binding was inhibited by gelatin, and fibrinogen showed some inhibitory activity in an assay where fibronectin binds to gelatin. When blood coagulates at low temperature, fibronectin binds to the fibrin clot (10), and it can be covalently linked to fibrin by the fibrin stabilizing factor (11). We found a marked reduction in the incorporation of fibronectin to the blood clot if gelatin was added to the blood before coagulation. These data strongly suggest that the same site on the fibronectin molecule is responsible for binding to collagen and fibrinogen, but that the site has a greater affinity for collagen sequences. We cannot exclude the possibility that small amounts of collagen contaminate the fibrinogen preparations we used. However, it would be difficult to explain the binding of fibronectin to the fibrin clots by assuming that the affinity of fibronectin for fibrinogen is actually due to binding to contaminating collagen. If fibronectin in tissues serves as a mediator of attachment of cells to the collagenous matrix, the significance of the fibrinogen binding could be to allow temporary binding of the cells to a fibrin scaffold in a wound.

Transformed cells lack fibronectin of their surface. This may contribute to their capacity to grow uninhibitedly and metastasize, because they would lack the supportive, and possibly growth inhibitory, attachment to the surrounding extracellular matrix.

Summary

Fibronectin, a fibroblast surface protein, was purified from human and chicken plasma and extracts of cultured chicken fibroblasts with affinity

chromatography on gelatin coupled to Sepharose particles. A fibronectin-like protein was also isolated from the plasma of Torpedo fish.

The collagen binding properties of fibronectin were studied with several genetically distinct collagens. Heat denatured types I, II, and III collagens were equal in their binding capacity and more active than the native collagens or A and B chains. Native type III collagen was more active than the other native collagens. Human and chicken fibronectins showed approximately the same pattern of specificity. Identical specificities were shown by the plasma and fibroblast forms of chicken fibronectin. Two cyanogen bromide peptides of the collagen $\alpha 1(II)$ chain, CB8 and CB12, derived from different parts of the chain, were active in fibronectin binding. A polymer of the tripeptide pro-gly-pro, and polyproline were inactive.

Fibronectin also binds to fibrinogen and fibrin. Comparison of this binding to collagen binding showed that fibrinogen inhibited binding of fibronectin to collagen, but was less active than native collagen. Two other fibrous proteins, tropoelastin and keratin, did not bind fibronectin. The binding of fibronectin to fibrinogen was inhibited by collagen and incorporation of fibronectin into blood clot in the cold was inhibited by gelatin. These results suggest that the binding of fibronectin to collagen and fibrinogen depends on the same binding site in the fibronectin molecule.

It is proposed that cell surface fibronectin mediates attachment of cells to the collagenous extracellular matrix and to a temporary fibrin matrix in a wound.

We thank Aulikki Pekkala for technical assistance, Dr. Sara Fuchs for a pro-gly-pro peptide sample, Dr. William Gray for elastin, and Dr. James Strauss for cultured fibroblasts.

Received for publication 13 February 1978.

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THE ROLE OF H-2-LINKED GENES IN HELPER T-CELL FUNCTION.

III. Expression of Immune Response Genes for Trinitrophenyl Conjugates of Poly-L(Tyr,Glu)-Poly-D,L-Ala--Poly-L-Lys in B Cells and Macrophages*

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For some years there has been intense interest in the problem of cellular expression of immune response (*Ir*)[1] genes. Initial experiments suggested that T cells alone were responsible for *Ir* manifestations since, for example, mice which were low responders to poly-L-(Tyr,Glu)-poly-D,L-Ala--poly L-Lys [(TG)-A--L] could make antibodies to this antigen coupled to methylated bovine serum albumin (1) and guinea pigs nonresponsive to the dinitrophenyl (DNP) hapten coupled to poly-L Lys could make antibodies to DNP coupled to other carriers (2).

The idea of T-cell expression of *Ir* genes was strengthened by later experiments which showed that T cells from high responder strains could divide in response to the appropriate antigen, whereas T cells from low responder strains could not (3–5). In an elegant experiment, Bechtol et al. (6) showed that low responder B cells could make antibody to (TG)-A--L in tetraparental low responder ↔ high responder mice. This result again suggested that *Ir* genes were not expressed in B cells. The role of macrophages (Mϕs), however, was not studied in any of these experiments.

More recently, however, *Ir* genes have been found to be associated with B cells and/or Mϕs. In the guinea pig, F_1, low responder × high responder T cells proliferated when cultured with high responder Mϕs pulsed with antigen, but not with low responder Mϕs (7, 8). Similarly, Katz et al. (9) have shown in the mouse that F_1, high responder × low responder T cells would help antibody responses of B cells from high responder mice, but not low responder mice to DNP-poly L-Glu, L-Lys, L-Tyr. Our work has shown that the *Ir* gene controlling cross-reaction between sheep erythrocytes (SRBC) and burro erythrocytes (BRBC) at the helper T-cell level is expressed at least by the B cell in vitro (10, 11).

The work of several laboratories (12–18) has suggested that at least three types of low responder animals can exist. The first type, exemplified by the response of mice of the *H-*

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* Supported by U. S. Public Health Service research grants AI-11558 and CA-11198, and American Cancer Society research grant IM-69.

‡ Recipient of an Established Investigatorship from the American Heart Association.

[1] *Abbreviations used in this paper:* B6AF$_1$, C57BL/6 × A F$_1$; B10, C57BL/10; BRBC, burro erythrocytes; BSS, balanced salt solution; CFA, complete Freund's adjuvant; DNP, dinitrophenyl(ated); GZ, beta-galactosidase; HRBC, horse erythrocytes; *Ir*, immune response; KLH, keyhole limpet hemocyanin; LPS, bacterial lipopolysaccharide; MHC, major histocompatibility complex; Mϕ, macrophage; PFC, plaque-forming cell; SRBC, sheep erythrocytes; (TG)-A--L, poly-L-(Tyr,Glu)-poly-D,L-Ala--poly-L-Lys; TNP, trinitrophenyl(ated).

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2^k haplotype to (TG)-A--L, is unable to respond because it has no functional B cells for this antigen, even though helper T cells are present (13–15). The second, exemplified by the response of mice of the H-2^f haplotype to (TG)-A--L, is unable to respond because it lacks helper T cells even though B cells responsive to this antigen are present (12). Finally, animals may lack both T cells and B cells responsive to a particular antigen, exemplified by the response of some H-2^s mice to (TG)-A--L and to poly(Phe,Glu)-poly-L-ala-poly-L-Lys (16–18).

In almost all cases studied, response or lack of response to a particular antigen maps in or close to the major histocompatibility complex (MHC) of the species in question (15, 19, 20). This is true even when separate genetic controls for B and T cells have been shown to exist, though there may be some non-MHC-encoded influence on at least B-cell responsiveness (18, 20).

Given the continuing controversy over the expression of *Ir* genes in T cells, B cells, or Mφs, we decided to dissect the response in vitro, where purified cell populations can more easily be separated and titrated together than in vivo. Direct plaque-forming cell responses to trinitrophenylated (TNP) (TG)-A--L in mouse spleen cell cultures were studied. These proved to have the same strain distribution of response as IgG responses to (TG)-A--L in vivo (19). When high responder × low responder F_1 cells were titrated with various combinations of B cells and Mφs of either the parental or F_1 *H-2* type, high responsiveness required the presence of at least high responder B cells, and, in the one case studied, high responder Mφs in the cultures, indicating the expression of *Ir* genes in both B cells and Mφs.

Materials and Methods

Mice. B10.A × C57BL10/Sn (B10) F_1, B10.M × B10 F_1, B10.M × B10.A F_1, B10.M, B10.A (4R), and CBA/J × C3H.SW/Sn F_1 were bred in our vivarium. Breeding mice for the B10.M, B10.A(4R), C3H.SW/Sn and B10.S strains were kindly provided us by Doctors M. Cherry and J. Stimpfling. All other mice were obtained from The Jackson Laboratory, Bar Harbor, Maine.

Cultures. Mouse spleen and lymph node cells were cultured by the methods of Mishell and Dutton (21), with some modifications (11).

Antigens. Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem, San Diego, Calif., and after dissolving in saline, was centrifuged at 78,000 *g* for 2 h (22). The pellet was then redissolved in saline and sterilized before storage. (TG)-A--L was purchased from Miles Laboratories Inc., Elkhart Ind. During these experiments two different lots were used (numbers MC3 and MC6), both of which had been previously tested by Miles and shown to give high titres of antiserum in responder, C3H.SW, and low titres of antisera in low responder, C3H/HeJ or C3H/DiSn, mice. The two lots proved indistinguishable in our hands.

TNP_{727}-KLH, assuming a mol wt of 8 × 10^6 for KLH, and TNP-β-galactosidase (TNP_{17}-GZ) were prepared by the method of Rittenberg and Pratt (23). TNP-(TG)-A--L was prepared similarly with the following exceptions. 50 mg of (TG)-A--L and 7 mg of trinitrobenzene sulfonic acid were mixed in 3 ml of cacodylate buffer (pH 6.9). After 30 sec, the reaction was stopped by the addition of excess glycylglycine. Samples having 2.6 and 3.2 TNP substitutions/100,000 daltons (TG)-A--L were used in these experiments. ^{125}I-TNP-(TG)-A--L was prepared by standard methods (24). 5 mg of TNP-(TG)-A--L was dissolved in 0.2 ml saline to which 0.1 ml 0.1 M sodium borate, pH 7.8, was added. To this, 7.5 μl ^{125}I-labeled sodium iodide was added. The mixture was vortexed and stood at room temperature for 1 min. 0.5 ml of 4 μM iodine chloride was then added, and the mixture was dialyzed extensively against saline followed by balanced salt solution (BSS).

TNP-*Escherichia coli* lipopolysaccharide (TNP-LPS) was prepared as described previously (25).

Immunizations. Mice were immunized to yield (TG)-A--L-specific T cells by injection of 100 μg (TG)-A--L in 40 μl complete Freund's adjuvant (CFA) in the base of the tail (26, 27). 7 days later, the periaortic and inguinal lymph nodes were removed and used as a source of T cells. The spleens of mice injected i.p. with 20–50 μg of KLH in CFA were used as a source of KLH-primed T cells. The spleens of mice injected i.p. 7 days previously with 1 μg TNP-LPS were used in most cases as a source of TNP-primed B cells (28). Such cells were primed in C3H/HeJ and C3H.SW/Sn mice by i.p. injection of 100 μg TNP-GZ in CFA 4–8 wk before use. In vitro immunization of cultures with TNP-KLH was by addition of 0.1 μg/ml TNP-KLH or as described below.

Antigen-Pulsed Mφs. Mφs were pulsed with antigen by modifications of the method of Pierce et al. (29). Briefly, the peritoneal cavities of normal mice were washed with ice-cold BSS. These washings were then centrifuged, and the cells were resuspended to 2 × 10^6/ml in ice-cold BSS. The relevant antigen, TNP-(TG)-A--L or TNP-KLH, was added to a final concentration of 100 μg/ml, and the mixture was incubated on ice for 1 h. The cells were than washed exhaustively with ice-cold BSS and counted before use in vitro. Experiments in which Mφs were pulsed with ^{125}I-TNP-(TG)-A--L showed that 10^6 Mφs bound ≅150 ng of antigen. Of this, ≅80% of the antigen was released during overnight culture. This was true for Mφs from both high responder and low responder strains. Although peritoneal washings clearly contain a heterogeneous population of cells, including lymphocytes and Mφs (30), the antigen-presenting cells will be referred to as Mφs throughout the rest of the paper since in our hands the functional cells have the following properties, all of which are characteristic of Mφs. They adhere to nylon fiber and Sephadex G-10. They are present in plastic adherent cells, >99% of which phagocytose latex particles. They are irradiation- and anti-T serum-plus-complement-resistant. Recent studies by others have, however, suggested that Mφs themselves may be heterogeneous, both in the Ia antigens they bear (31, 32), and in their biological and biochemical properties (32, 33). Yamashita and Shevach (32) have reported that it is the Ia-positive subpopulation of Mφs which is most efficient at antigen presentation in their experiments. We have not characterized the active subpopulation in our experiments.

T and B Cells. (TG)-A--L-specific T cells were prepared from the periaortic and inguinal lymph nodes of immunized mice (26, 27), KLH-specific T cells were isolated from the spleens of KLH-immunized mice. In both cases, the cells were passed through nylon fiber columns (11, 34, 35) to remove B cells, Mφs, and other nylon fiber-adherent cells before use in vitro. B cells were isolated from spleen cell suspensions from TNP-primed mice by treatment of the cells with anti-T serum and complement (36). In some experiments it was also necessary to remove Mφs from the B-cell populations. In these cases the B cells were Mφ-depleted by passage through Sephadex G-10 columns (37) before treatment with anti-T serum and complement.

Direct Plaque-Forming Cell (PFC) Assay. After 4 days of culture, two or three identical culture wells were pooled and assayed in duplicate for direct anti-TNP PFC using the slide modification (21) of the Jerne hemolytic plaque assay. Parallel determinations were made using TNP-horse erythrocytes (TNP-HRBC) and HRBC, and the difference was recorded as the number of anti-TNP PFC. For these assays, lightly conjugated TNP-HRBC were prepared by the method of Rittenberg and Pratt (23), as modified by Kettman and Dutton (38). HRBC from a single animal were obtained from the Colorado Serum Co., Denver, Colo.

Assay of Helper T-Cell Activity. Helper T-cell activity was titrated as previously described (28). Culture wells were set up containing 3 × 10^6 TNP-primed B cells with or without Mφs from the appropriate strain of mouse. For TNP-(TG)-A--L responses, 10^5–2 × 10^5 TNP-(TG)-A--L-pulsed Mφs were added to each culture. For TNP-KLH responses, either 10^5–2 × 10^5 TNP-KLH-pulsed Mφs were added to each culture, or the culture medium was supplemented with TNP-KLH to a final concentration of 0.1 μg/ml. Varying numbers of T cells primed to the appropriate antigen were then added to the cultures. A plot of anti-TNP PFC/culture vs. the number of helper cells added yielded a titration with an initially linear slope. The least squares line was fitted to the initial points, and the slope of the line was taken as the activity of the helper population (Fig. 1).

Results

Conditions for in Vitro Anti-TNP-(TG)-A--L Responses. Conditions were established under which we could observe anti-TNP-(TG)-A--L responses in vitro. In preliminary experiments we found that the conditions which we had previously used to study anti-TNP-KLH responses (28) were not sufficient to generate in vitro anti-TNP-(TG)-A--L responses. A number of alterations were required, as described below.

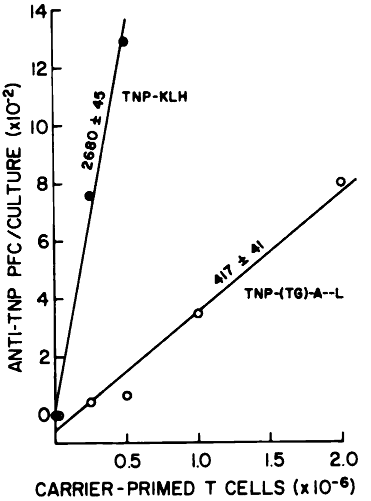

FIG. 1. Titration of (TG)-A--L-specific and KLH-specific helper T cells. Three B6AF$_1$ mice were primed with (TG)-A--L, and two with KLH. Seven days later, T cells were prepared from these mice and titrated for their helper activity in anti-TNP-(TG)-A--L responses (O) and anti-TNP-KLH responses (●), respectively, as described in Materials and Methods. The number of anti-TNP PFC/culture on day 4 is plotted vs. the number of (TG)-A--L-primed or KLH-primed T cells/culture. The initial slope ± SE of each titration line is also shown.

T cells isolated from the spleens of mice immunized i.p. with (TG)-A--L in CFA were not effective as helper cells in this response. This problem was solved by using T cells isolated from the periaortic and inguinal lymph nodes of mice immunized in the base of the tail with (TG)-A--L in CFA as described in Materials and Methods, and by others (26, 27). Such preparations were rich in (TG)-A--L-specific helper T cells.

We were unsuccessful in obtaining responses to TNP-(TG)-A--L when the antigen was added in soluble form to our cultures. This has been a common result when using soluble, relatively small antigens in our laboratory (39). This problem was solved by adding the antigen to cultures bound to peritoneal Mφs (Materials and Methods).

As had been our previous experience with TNP-protein antigens in vitro (28), a vigorous anti-TNP-(TG)-A--L response in vitro required the use of TNP-primed B cells. These were prepared from the spleens of mice primed either with TNP-LPS or, in the case of mice of the C3H background, with TNP-GZ in CFA.

After these conditions had been satisfied, good anti-TNP-(TG)-A--L responses occurred in our cultures. For example, in Fig. 1 the anti-TNP-(TG)-A--L response of C57BL/6 × A/J (B6AF$_1$) B cells and Mφs is plotted as a function of the number of (TG)-A--L-primed B6AF$_1$ T cells added to the cultures. Anti-TNP PFC numbers rose linearly as the numbers of (TG)-A--L-primed T cells in cultures were increased. In a control experiment, these same B cells and Mφs were shown to respond well to TNP-KLH in the presence of increasing numbers of KLH-primed B6AF$_1$ T cells. Other experiments, not shown here, demonstrated that (TG)-A--L priming of the T cells was required, and that TNP-

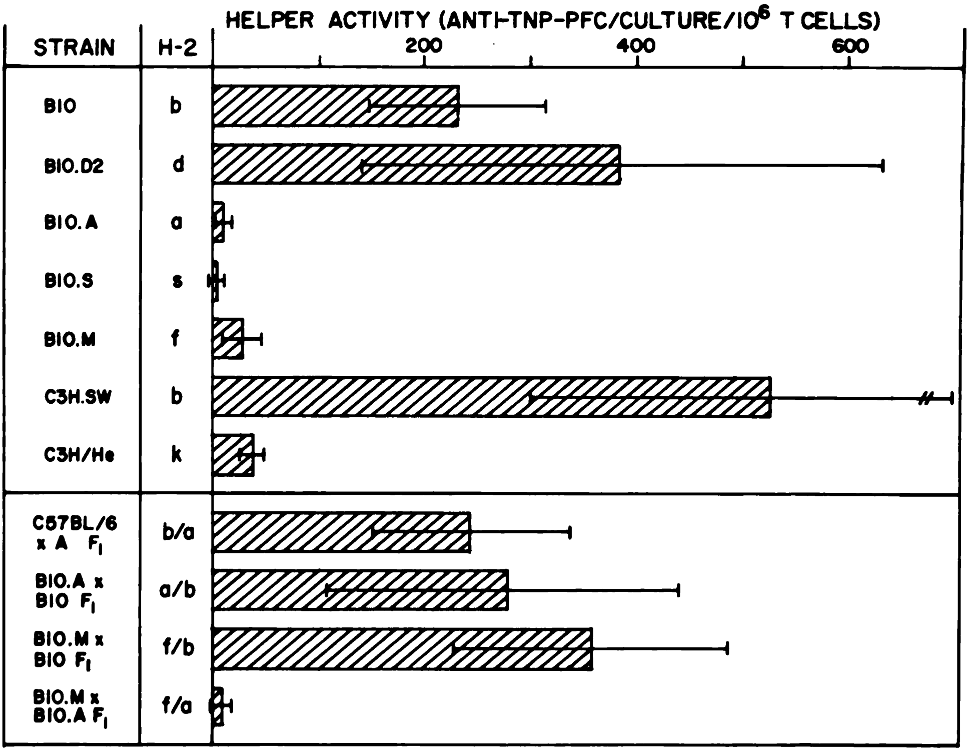

FIG. 2. Strain distribution of response to TNP-(TG)-A--L. T cells from different strains of mice were titrated for their helper activity in anti-TNP-(TG)-A--L responses as described in Materials and Methods and in Fig. 1. Shown here are the results of 38 separate determinations in which each strain was tested at least three times. For each titration, the number of anti-TNP PFC/culture was plotted vs. the number of T cells added, and the initial slopes were determined by the least squares method. Slopes for all determinations with a given strain were averaged and are shown ± SEM.

(TG)-A--L had to be present in the cultures for anti-TNP-(TG)-A--L responses to occur.

Strain Distribution of in Vitro Responses to TNP-(TG)-A--L. The ability of cells from different strains of mice to respond to TNP-(TG)-A--L was measured by titrating the ability of (TG)-A--L-primed T cells from each strain to stimulate the direct PFC response to TNP-(TG)-A--L of TNP-primed B cells and Mϕs from the same strain. As a control, KLH-primed T cells were also prepared in each strain and tested for their ability to stimulate an anti-TNP-KLH response in the same B cell and Mϕ population. The averaged results of a number of experiments on the strains so far tested are shown in Fig. 2. All strains responded well to TNP-KLH (results not shown). By contrast, B10, B10.D2/nSn, and C3H.SW mice all responded well to TNP-(TG)-A--L, but B10.A, B10.S, B10.M, and C3H/He mice responded poorly, if at all. Of the F_1 mice tested, B10.A × B10 F_1, B6AF$_1$, and B10.M × B10 F_1 all gave large responses, whereas B10.M × B10.A F_1 mice did not respond. These results are in agreement with the reports of in vivo IgG responses to (TG)-A--L (19) and with some of the published reports of in vivo IgM responses to (TG)-A--L (20, 40, 41), and in vitro IgM responses to TNP-(TG)-A--L (42), with exceptions discussed later.

TABLE I
Mapping of Ir Gene(s) for Anti-TNP-(TG)-A--L Responses in Vitro

Strain	*H-2* subregions*									Anti-TNP-(TG)-A--L response (PFC/10^6 T cells)‡
	K	*I-A*	*I-B*	*I-J*	*I-E*	*I-C*	*S*	*G*	*D*	
B10.A(5R)	b	b	b	k	k	d	d	d	d	685 ± 351
B10.A(4R)	k	k	b	b	b	b	b	b	b	8 ± 7
B10.A(2R)	k	k	k	k	k	d	d	d	b	4 ± 5
B10.A	k	k	k	k	k	d	d	d	d	8 ± 6§
B10.D2	d	d	d	d	d	d	d	d	d	385 ± 246§
B10	b	b	b	b	b	b	b	b	b	228 ± 88§

* Haplotype data from references 50-52.

‡ Average ± SEM of between 3 and 6 separate determinations.

§ Data from Fig. 1.

Experiments were performed in B10 congenic mice with recombinant *H-2* haplotypes to allow preliminary mapping of the *Ir* gene(s) controlling the in vitro direct PFC response to TNP-(TG)-A--L. As shown in Table I, B10.A(5R) mice responded well to the antigen, but B10.A(4R) and B10.A (2R) responded poorly, suggesting that the gene(s) controlling this phenomenon map in the *K*, *I-A* end of the *H-2* complex. This location for genes controlling in vivo and in vitro responses to (TG)-A--L has already been well established by others (15, 19, 42).

Expression of the Ir Gene(s) in B Cells. Having shown that the in vitro response to TNP-(TG)-A--L was under the control of *Ir* gene(s), we wished to determine which cell type(s) were expressing the gene(s) in vitro: T cells, B cells, or Mϕs. Since these cell types cannot be taken from different unrelated mice and mixed in vitro without generating mixed lymphocyte reactions and complicating allogeneic effects, we designed these experiments along the lines we have previously described (10, 11, 43, 44). Thus, (TG)-A--L-primed T cells were obtained from F_1 mice, the cross between high responder and low responder parents. These T cells were then titrated for their ability to stimulate anti-TNP-(TG)-A--L responses of B cells and Mϕs obtained from congenic mice identical at *H-2* with either the high responder or low responder parent. TNP-(TG)-A--L was added to the cultures bound to either high responder or low responder Mϕs. F_1 T cell and congenic B cell and Mϕ donors were also selected such that no anti-*Mls* activity (45) would be obtained. This protocol permitted cultures to be set up with cells from mice differing at *H-2*, and it eliminated undesirable allogeneic effects since the F_1 T cells were incapable of recognizing the *H-2* or *Mls* antigens of the B cells and Mϕs. We hoped that this protocol would determine whether high responder F_1 T cells were sufficient for a high anti-TNP-(TG)-A--L response, or whether high responder B cells and/or Mϕs were also required.

Three examples of this type of experiment are shown in Fig. 3. The results in Fig. 3a show that when (TG)-A--L-immunized B6AF$_1$ ($H\text{-}2^b \times H\text{-}2^a$) T cells were titrated for their ability to help anti-TNP-(TG)-A--L responses of B10 ($H\text{-}2^b$, high responder) or B10.A(2R) ($H\text{-}2^{h2}$, low responder) cells, high responses were obtained with B10 cells and low responses with B10.A(2R) cells, irrespective of the type of Mϕ bearing antigen in the cultures. Identical experiments

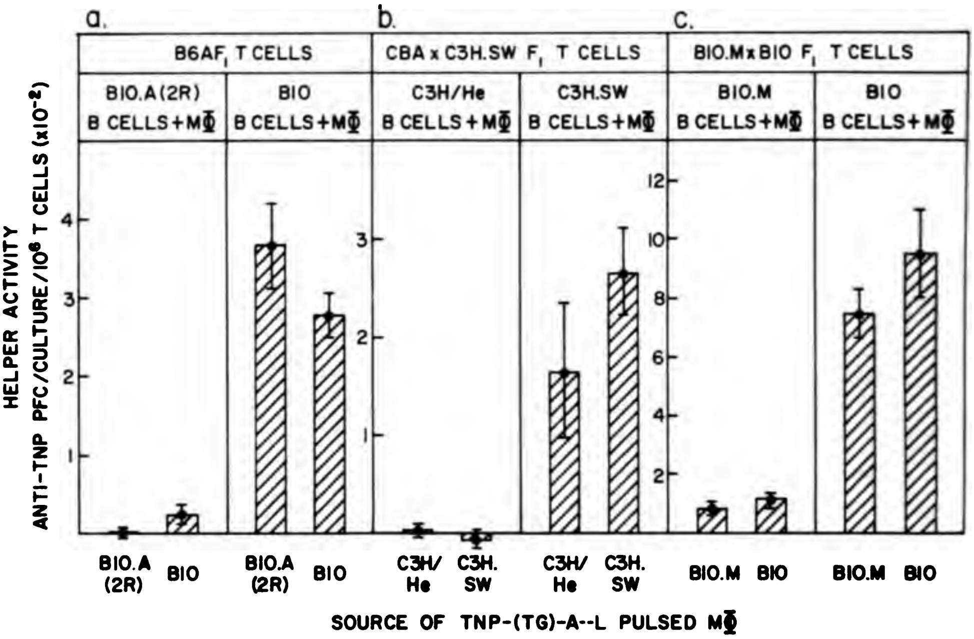

FIG. 3. Expression of Ir genes in B cells. F$_1$, high responder × low responder mice were primed with (TG)-A--L. Their T cells were subsequently titrated for helper activity in anti-TNP-(TG)-A--L responses of B cells and Mφs from high or low responder mice congenic with the parents of F$_1$ at *H-2*. TNP-(TG)-A--L was added to cultures bound to high or low responder Mφ. Slopes ± SE of titrations for different T-cell, B-cell and Mφ, and antigen-bound Mφ preparations are shown, obtained in representative experiments. Each experiment was performed three times. Results are shown for three different strain combinations. (a) B6AF$_1$ T cells, B10.A(2R) low responder, and B10 high responder B cells and Mφs; (b) CBA × C3H.SW F$_1$ T cells, C3H/He low responder, and C3H.SW high responder B cells and Mφs; (c) B10.M × B10 F$_1$ T cells, B10.M low responder, and B10 high responder B cells and Mφs.

were performed with similar results using CBA/J × C3H.SW ($H\text{-}2^{k}$ × $H\text{-}2^{b}$) F$_1$ (TG)-A--L-primed T cells and B cells and Mφs from C3H/HeJ ($H\text{-}2^{k}$, low responder) or C3H.SW ($H\text{-}2^{b}$, high responder) mice (Fig. 3b). Another set of experiments was performed with B10.M × B10 F$_1$ ($H\text{-}2^{f}$ × $H\text{-}2^{b}$) (TG)-A--L-primed T cells and B cells and Mφs from B10.M ($H\text{-}2^{f}$, low responder) or B10 ($H\text{-}2^{b}$, high responder) mice, again with qualitatively similar results (Fig. 3c). It should be noted, however, that the anti-TNP-(TG)-A--L responses of B10 B cells and Mφs, when stimulated by B10.M × B10 F$_1$ T cells, were the highest of all the strain combinations tested, and that the responses of B10.M B cells and Mφs, when stimulated by the same T cells, were much smaller than these, but appreciable by comparison with other strain combinations. Each of these experiments has been performed three times with similar results.

Thus, in the three cases examined, the presence of high responder F$_1$ T cells was not a sufficient condition for high response. In each case, cultures also had to contain high responder B cells for good anti-TNP-(TG)-A--L responses to occur, indicating the expression of *Ir* genes in B cells. We were tempted to conclude from our experiments that the *Ir*-type of the antigen-presenting Mφ was irrelevant in the response. Some subsequent control experiments using ^{125}I-TNP-(TG)-A--L, however, indicated that this conclusion was not justified. In these experiments both high and low responder Mϕs were shown to take up approximately the same amount of TNP-(TG)-A--L during our pulsing procedure. More importantly, both types released ≅80% of this bound antigen within 24 h of culture. Thus, in the experiments shown in Fig. 3, there was a possibility of antigen-transfer from the original antigen-bearing Mϕs to those introduced with the B-cell preparation. The high responses obtained when F_1 T cells were cultured with high responder B cells and Mϕs and antigen-pulsed low responder Mϕs might have been due to antigen-transfer to the high responder Mϕs. The problem of *Ir*-gene expression in Mϕs in our cultures was, therefore, still unanswered. This issue was addressed in a further set of experiments described in the following section.

Two types of controls were performed for these mixing experiments. First, F_1 (TG)-A--L-primed T cells were titrated into cultures containing splenic B cells and Mϕs from one strain, and peritoneal Mϕs from the other strain, in the absence of antigen. TNP PFC/culture/10^6 T cells were always <2 under such circumstances, suggesting that no nonspecific stimulation of anti-TNP PFC responses was resulting from the mixing of peritoneal cells from one strain with B cells and Mϕs from the other, in the presence of T cells. In other control experiments, low responder B cells were shown to respond to TNP coupled to unrelated antigens by testing their response to TNP-KLH bound to high responder or low responder Mϕs in the presence of F_1 KLH-primed T cells (results not shown).

Expression of Ir Gene(s) in B Cells and Mϕ. To discover whether *Ir* genes were being expressed in Mϕs as well as B cells in our cultures, splenic B cells had to be depleted of Mϕs by passage over Sephadex G-10 columns. Such a maneuver prevented possible antigen transfer from the Mϕs on which it was added to cultures to Mϕs in our splenic B-cell populations. Thus B10.A or B10 B cells were depleted of Mϕs, TNP-(TG)-A--L was added to cultures bound to either B10 or B10.A Mϕs, and B6AF_1 (TG)-A--L-primed T cells were titrated into the cultures. The results of a typical experiment of three are shown in Fig. 4 a. As in the previous experiments, B10.A B cells did not respond to TNP-(TG)-A--L in the presence of B6AF_1 (TG)-A--L-primed T cells whether the antigen was added to cultures bound to B10.A or B10 Mϕs. B10 B cells responded to TNP-(TG)-A--L in the presence of helper T cells if the antigen was added to cultures on the surface of B10 Mϕs but, unlike the previous experiment, not if antigen was added on the surface of B10.A Mϕs. These results suggested that *Ir* genes were being expressed in vitro by both B cells and Mϕs. Again, in control experiments both the B10 and B10.A Mϕs and B cells were shown to be functional in anti-TNP-KLH responses in the presence of KLH-primed F_1 T cells.

These results could also be explained by a theory in which *Ir* genes are expressed only in B cells or Mϕs in vitro, but *H-2* compatibility is required for good B cell-Mϕ cooperation. Thus, *Ir* genes might be expressed in Mϕs only, for example, but B10 Mϕs would be unable to cooperate with B10.A B cells because the two are histoincompatible. To eliminate this explanation, experiments were

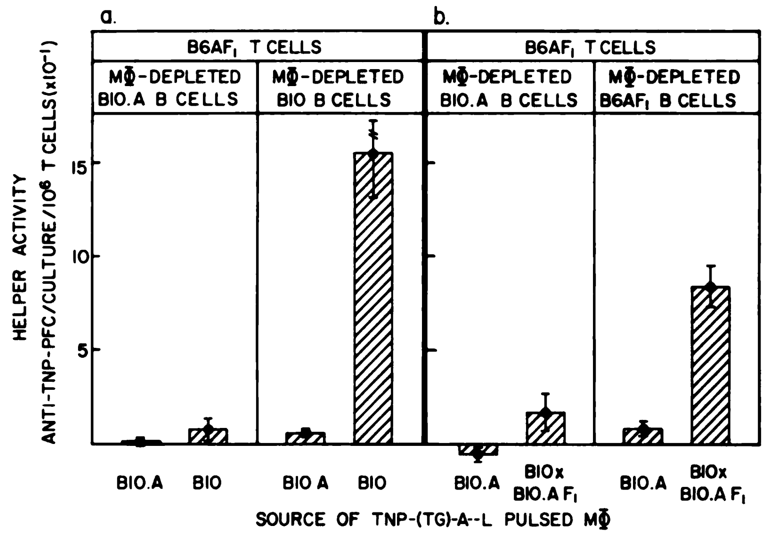

FIG. 4. Expression of Ir genes in B cells and Mϕs. (a) B6AF$_1$ mice were primed with (TG)-A--L and their T cells were subsequently titrated for helper activity in anti-TNP-(TG)-A--L responses of Mϕ-depleted B cells from B10.A, low responder, or B10, high responder animals. Antigen was added to cultures bound to B10.A or B10 Mϕs. The initial slopes ± SE obtained in a representative experiment of 3 are shown. (b) As in (a), except that Mϕ-depleted B cells were obtained from B10.A and B6AF$_1$ animals and antigen-pulsed Mϕ were obtained from B10.A and B10 × B10.A F$_1$ animals.

performed in which Sephadex G-10-passed B6AF$_1$ or B10.A B cells were tested for their response to TNP-(TG)-A--L bound to B10.A or B10 × B10.A F$_1$ Mϕs in the presence of B6AF$_1$ (TG)-A--L-primed T cells. In this experiment, B cells and Mϕs were always at least semihistocompatible, thus, *H-2*-controlled restrictions on B cell-Mϕ interactions should not occur. The results of one such experiment is shown in Fig. 4b. B10.A B cells did not respond to TNP-(TG)-A--L , even if the antigen was added to cultures bound to B10 × B10.A F$_1$, semihistocompatible, responder Mϕs. Conversely, the response of B6AF$_1$ B cells to TNP-(TG)-A--L in the presence of F$_1$ helper T cells ocurred only if antigen was added to the cultures bound to B10 × B10.A F$_1$ Mϕs, not if bound to B10.A Mϕs. These results were repeated in two other experiments. These results show again that *Ir* gene(s) controlling (TG)-A--L responses are being expressed by both B cells and Mϕs in vitro, and that our results cannot be explained by lack of interaction between histoincompatible B cells and Mϕs.

Discussion

The problem of cellular expression of *Ir* genes in antibody responses seems to be particularly difficult to solve, since at one time or another B cells, Mϕs, and T cells have all been implicated. Proliferation experiments in guinea pigs and mice, for example, have shown that at least Mϕs, and possibly also T cells, may express the genes (5, 7, 8). Other experiments have shown that B cells may also be involved (9-11). The problem is not simplified if the experiments considered are confined to those involving only one antigen, (TG)-A--L. McDevitt et al. (4), Bechtol et al. (6) have shown that $H\text{-}2^{k}$-bearing B cells in $H\text{-}2^{k} \leftrightarrow H\text{-}2^{b}$

tetraparental mice are not defective in their ability to respond to this antigen, although T cells and/or Mϕs from mice of the $H\text{-}2^k$ haplotype are apparently nonresponsive (3). On the other hand, the exporiments of Munro and Taussig (12), Litchenberg et al. (14), Erb and Feldmann (13), and Howie and Feldmann (46) suggest that $H\text{-}2^k$ B cells are defective and $H\text{-}2^k$ T cells are functional in anti-(TG)-A--L responses.

According to Munro and Taussig (12), Lichtenberg et al. (14), Mozes et al. (16), and Munro et al. (18), however, in other strains of mice the defect in response to (TG)-A--L may lie in either the T cells alone (B10.M, A.CA, $H\text{-}2^f$; A.SW, $H\text{-}2^s$), or in the T cells and B cells (S.JL: $H\text{-}2^s$). The role of the Mϕ was not studied in these experiments, but it has been suggested by others that so-called T-cell-defective mice did in fact contain deficient Mϕs.

Since it is very difficult to deplete mice of Mϕs, it seemed reasonable to us to examine the possible expression of *Ir* genes for (TG)-A--L by B cells and Mϕ in vitro. Thus, we established conditions in vitro under which antibody responses to TNP coupled to (TG)-A--L could be obtained. Our direct PFC responses were secondary since both B cells and T cells were primed to antigen. When the response of cells from different strains of mice to TNP-(TG)-A--L was measured, the pattern of response was as predicted from the in vivo distribution of IgG responses to (TG)-A--L (19) and a recently published survey of IgM primary responses to TNP-(TG)-A--L in vitro (42). The one exception to this was our observation that B10.D2 mice were high responders to TNP-(TG)-A--L, whereas in vivo results predict that they should be intermediate. There is some controversey in the literature over *Ir* restriction of IgM responses to (TG)-A--L (20, 40–42). Results seem to depend on the antigen and conditions used. Our system, however, detects *Ir*-controlled differences in direct PFC responses.

We were somewhat surprised to find that mice of the $H\text{-}2^a$ and $H\text{-}2^k$ haplotypes were low responders to TNP-(TG)-A--L, since the experiments of Howie and Feldmann (46) show that they should be high responders. Singer et al. (42), who also show that $H\text{-}2^a$ and $H\text{-}2^k$ mice are nonresponders to TNP-(TG)-A--L, have suggested that this anomalous result of Howie and Feldmann (46) results from their methods of preparing antigen. We were also disappointed to note that B10.M × B10.A F_1 mice were low responders to TNP-(TG)-A--L in our hands. Munro and Taussig (12) and Munro et al. (18) originally showed that gene complementation occurred in this F_1 combination, a result which is very intriguing. In subsequent experiments, McDevitt (41) and Munro and Taussig (47) have been unable to repeat the original finding, in agreement with our result.

We concluded, however, that our in vitro IgM secondary responses were measuring the presence of an *Ir* gene(s) with a strain distribution identical to that identified by others in vivo and in vitro. Moreover, this gene(s) mapped in the same part of the *H-2* complex as gene(s) controlling other (TG)-A--L responses, viz *K*, *I-A*.

Having established this, we set up experiments along the lines originally published by Katz et al. (9) and Shevach and Rosenthal (7), and more recently ourselves (10, 11). (TG)-A--L primed T cells were obtained from F_1 mice, the cross between high and low responder parents. These T cells were titrated for their ability to help anti-TNP-(TG)-A--L responses of B cells and Mϕs of either parental *H-2* type. Three different strain combinations were studied. In every case, low responder B cells (B10.A(2R), $H\text{-}2^{h2}$; C3H/HeJ, $H\text{-}2^{k}$; B10.M, $H\text{-}2^{f}$) were unable to respond in the presence of active T cells and Mϕs of the high responder type. We concluded from these experiments that *Ir* genes were being expressed at least by B cells in all three low responder strains studied here.

We wished to test for the expression of *Ir* genes by Mϕs in antibody responses. To do this, Mϕs were removed from our B-cell populations to prevent antigen transfer, and F_1 cells were titrated into cultures containing B10.A ($H\text{-}2^{a}$, low responder) or B10 ($H\text{-}2^{b}$, high responder) B cells. Antigen was added on B10.A or B10 Mϕs. B10.A B cells did not respond to TNP-(TG)-A--L, regardless of the Mϕ type in vitro. B10 B cells responded only when B10 Mϕs were added. Using F_1 Mϕs, we were able to show that the lack of response of B10.A B cells was not due to *H-2* differences between the B cells and Mϕs.

We therefore concluded that B10.A mice were low responders to TNP-(TG)-A--L because they lacked both functional B cells and functional Mϕs for this antigen. Of the other strains studied, C3H/HeJ ($H\text{-}2^{k}$), B10.A(2R), and B10.M ($H\text{-}2^{f}$) lacked at least functional B cells. Our finding that B10.M B cells are nonfunctional is in direct contradiction to the results of Munro and Taussig (12), who suggest that B10.M mice should contain functional B cells and nonresponsive T cells for (TG)-A--L. This is a contradiction we are at present unable to resolve except by pointing out that the conditions of our experiments are vastly different.

Although the antigen presenting cells in our cultures have been identified as Mϕs by several criteria (Materials and Methods), we have not characterized the subpopulation of Mϕs which are active in our experiments. Since it has been shown by other investigators that it is the Ia-positive subpopulation of Mϕs which are most active in antigen presentation (32), our future experiments will be designed to test whether it is the Ia-positive Mϕs which differ in their ability to present TNP-(TG)-A--L between low responder and high responder strains of mice.

None of the experiments described in this paper examine the question of *Ir* gene expression in T cells. Thus, all the low responder strains we have tested may also contain nonfunctional T cells for (TG)-A--L responses. Our future experiments will be designed to tackle this question.

The results which we present here are consistent with our previous findings (11) that the *Ir* gene which controls the ability of helper T cells to respond to a cross-reacting determinant on SRBC and BRBC is expressed at least at the level of the B cell.

A number of models have been proposed to explain how MHC-linked *Ir* genes can control the activity of helper T cells even though they are expressed in B cells and Mϕ (12, 48, 49). At present, we favor the associative or dual recognition hypothesis in which the specificity of helper T cells is determined by the simultaneous recognition of antigen and *Ir*-gene products on either the Mϕ or B-cell surface (49). Thus, B cells and Mϕs in high responder strains would possess, and in low responder strains would lack the appropriate I-region encoded molecule which could be recognized in association with the antigen. The attraction of this model is that it is consistent with a large body of evidence in the literature (13, 29, 48, 49), including work from this laboratory (11, 43, 44) concerning the interaction of helper T cells with antigen.

Summary

Using lymph node T cells from poly-L(Tyr,Glu)-poly-D,L-Ala--poly-L-Lys [(TG)-A--L]-primed animals and B cells from animals primed with trinitrophenylated (TNP) protein or lipopolysaccharide, we have obtained anti-TNP-(TG)-A--L direct plaque-forming responses in vitro. Response to this antigen was shown to be controlled by the *H-2* haplotype of the animal studied. The strain distribution of in vitro response was very similar to that previously reported by others for in vivo secondary IgG responses to (TG)-A--L.

We investigated the cell types expressing the *Ir* gene(s) for (TG)-A--L in our cultures. F_1, high responder × low responder mice were primed with (TG)-A--L. Their T cells were active in stimulating anti-TNP-(TG)-A--L responses of high responder but not low responder B cells and macrophages (Mϕ), even though both preparations of B cells and Mϕ were obtained from mice congenic at *H-2* with one of the parents of the F_1. For three low responder strains tested, of the $H\text{-}2^{h2}$, $H\text{-}2^{k}$, and $H\text{-}2^{f}$ haplotypes, the anti-TNP-(TG)-A--L response of low responder B cells and Mϕs in the presence of high responder, F_1 T cells could not be improved by the addition of high responder, antigen-bearing Mϕs to the cultures. In one strain of the $H\text{-}2^{a}$ haplotype, it was shown that neither the B cells nor Mϕs could be functional in anti-TNP-(TG)-A--L responses. Our results therefore suggested the *Ir* genes for anti-TNP-(TG)-A--L responses were expressed at least in B cells in all the low responder strains we studied, and, in mice of the $H\text{-}2^{a}$ haplotype, in Mϕs too.

We thank Lee Harwell and George Berry for their excellent technical assistance. We thank the Animal Tumor Research Facility and the Biostatistics Facility of the University of Rochester Cancer Center for their help in supplying tumor cells for antiserum and complement absorbtion, and in developing computer programs for the statistical handling of our data, respectively.

Received for publication 28 December 1977.

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CELLULAR AND GENETIC CONTROL OF ANTIBODY RESPONSES IN VITRO

III. Immune Response Gene Regulation of Accessory Cell Function

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Antibody responses to a variety of antigens, including certain synthetic polypeptides such as poly-L-(Tyr,Glu)-poly-D,L-Ala--poly-L-Lys [(T,G)-A--L][1] and poly-L-(His,Glu)-poly-D,L-Ala--poly-L-Lys [(H,G)-A--L] are regulated in the mouse by immune response (*Ir*) genes which are located within the murine major histocompatibility complex (*H-2*) (1). Since these *Ir* gene-controlled antibody responses require interactions among several cell populations, it is important for an understanding of the mode of action of *Ir* genes to determine the cell type(s) which express *Ir* gene function. Despite much research effort, the identity of such cell type(s) remains controversial (2, 3).

The cell type(s) involved in *Ir* gene control has been most extensively studied in the responses to (T,G)-A--L and (H,G)-A--L of *H-2*^{a}, *H-2*^{b}, and *H-2*^{k} mouse strains. Some investigators have implicated T lymphocytes as the cell type which expressed *Ir* genes in these responses since: (*a*) the ability of responder *H-2*^{b} strains to produce (T,G)-A--L-specific IgG antibodies and to generate (T,G)-A--L-specific memory was abolished by neonatal thymectomy (4), and (*b*) B lymphocytes from nonresponder *H-2*^{k} strains were capable of producing (T,G)-A--L-specific antibodies when immunized with (T,G)-A--L complexed to methylated bovine serum albumin (5) or when stimulated by nonspecific graft-versus-host reactions (6). Conversely, other investigators have implicated B lymphocytes as the cell type which expresses *Ir* genes in these same responses since: (*a*) limiting dilution experiments demonstrated that reactive (T,G)-A--L-specific bone marrow cells, not thymocytes, were the limiting cell type in nonresponder *H-2*^{k} strains (7), and (*b*) bone marrow cells from (T,G)-A--L nonresponder *H-2*^{a} and *H-2*^{k} strains, and from (H,G)-A--L nonresponder *H-2*^{b} strains, were unable to absorb or to respond to antigen-specific T-cell replacing factors, even though educated thymocytes from these nonresponder strains were competent to produce such (T,G)-A--L- and (H,G)-A--L-specific factors (8, 9). However, the possibility was not excluded in these previous studies that *Ir* genes were expressed in cells other than T or B cells which were required for the generation of antibody responses, i.e. accessory cells.

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; C′, guinea pig complement; FACS-II, fluorescence-activated cell sorter; Fl-F(ab′)$_2\alpha$Fab, fluorescein isothiocyanate-conjugated rabbit F(ab′)$_2\alpha$anti-mouse Fab; (H,G)-A--L, poly-L-(His,Glu)-poly-D,L-Ala--poly-L-Lys; *Ir*, immune response; KLH, keyhole limpet hemocyanin; PFC, plaque-forming cells; RαMB, rabbit anti-mouse brain serum; sIg, surface immunoglobulin; SRBC, sheep erythrocytes; (T,G)-A--L, poly-L-(Tyr,Glu)-poly-D,L-Ala--poly-L-Lys; TNP, trinitrophenyl.

Recently, an in vitro primary IgM antibody-forming cell assay using normal spleen cells was characterized (10, 11). This assay could potentially be utilized to determine which cell type(s) express *Ir* genes, since responses to trinitrophenyl (TNP) conjugates of (T,G)-A--L and (H,G)-A--L in this system were under autosomal dominant, *H-2*-linked *Ir* gene control, and required the presence of adherent accessory cells, T cells, and B cells (10, 11). In this report, the possibility was examined that accessory cells participate in the *Ir* gene control of primary plaque-forming cell (PFC) responses to TNP-(T,G)-A--L and TNP-(H,G)-A--L.

Materials and Methods

Animals. C57BL/10 (B10), B10.A, (B10 × B10.A)F_1, B10.A(4R), B10.A(5R), A/J, A.BY, and (A/J × A.BY)F_1 mice of both sexes were obtained from The Jackson Laboratory, Bar Harbor, Maine. All mice were used at 6–12 wk of age, and were sex matched in each experiment.

Antigens. (T,G)-A--L (lot MC-3; Yeda Research and Development Co., Ltd., Rehovot, Israel), (H,G)-A--L (the generous gift of Dr. Edna Mozes, Weizmann Institute of Science, Rehovot, Israel), and Keyhole limpet hemocyanin (KLH) (lot 530195; Calbiochem, San Diego, Calif.) were conjugated with 2,4,6-trinitrobenzene sulfonate (Pierce Chemical Co., Rockford, Ill.) as previously described (10). The degree of TNP modification was 8 TNP groups per 100,000 daltons (T,G)-A--L, 5 TNP groups per 100,000 daltons (H,G)-A--L, and 12 TNP groups per 100,000 daltons KLH. The final concentration used in culture was the optimum concentration for each antigen preparation and was 100–250 μg/ml TNP-(T,G)-A--L, 100–250 μg/ml TNP-(H,G)-A--L, and 5–10 μg/ml TNP-KLH (10, 11).

Preparation of Cells

SEPHADEX G-10 PASSAGE OF SPLEEN CELLS. Spleen cells were passed sequentially over two Sephadex G-10 columns (lot 9067; Pharmacia, Inc., Piscataway, N. J.) by a modification of the method of Ly and Mishell (12) described elsewhere (10). Total cell recovery after two sequential Sephadex G-10 passages ranged from 40 to 60%. In a series of 16 consecutive experiments, the percentage of B (surface Ig$^+$[sIg$^+$]) cells (10) in the cell population was not significantly affected by the Sephadex G-10 passages (51.1 ± 2.4% before G-10 passage and 53.0 ± 2.8% after G-10 passage); the percentage of T (rabbit anti-mouse brain$^+$[RαMB$^+$]) cells (10) was somewhat increased (28.5 ± 1.8% before G-10 passage and 39.4 ± 2.8% after G-10 passage). Conversely, Sephadex G-10 passage markedly reduced the number of phagocytic cells as measured by latex ingestion (6.9 ± 1.2% before G-10 passage to 0.73 ± 0.1% after G-10 passage) (13).

PREPARATION AND CHARACTERIZATION OF SPLEEN ADHERENT CELLS (SAC). The preparation of 2-h glass-adherent spleen cells has been described in detail previously (10, 13). All adherent cell populations were sequentially treated with anti-Thy 1.2 serum (lot 231-72-6; Litton Bionetics, Kensington, Md.), guinea pig complement (C′) (Flow Laboratories, Inc., Rockville, Md.), and irradiation with 1,000 R. The adherent cells were then precultured overnight on a roller drum at 37°C and added to cell cultures the next day (10).

The final viable cell recovery of adherent, radiation-resistant, anti-Thy 1.2-treated cells was $\cong$1% of the initial spleen cell population and, in a large number of experiments, consisted of 50–80% phagocytic cells, 8–15% nonphagocytic sIg$^+$ cells, no detectable (<0.3%) RαMB$^+$ cells, and 15–25% nonphagocytic cells negative for latex, sIg, and RαMB ("null" cells).

DEPLETION OF SIG$^+$ CELLS FROM THE SPLEEN ADHERENT CELL POPULATION USING THE FLUORESCENCE-ACTIVATED CELL SORTER (FACS-II). Spleen adherent cells were prepared as above but the medium lacked phenol red to diminish autofluorescence. After the overnight preincubation, 5 × 10^6 adherent cells were resuspended in 0.05 M phosphate-buffered saline, pH 7.2, containing 2%

bovine serum albumin (BSA), and reacted with 0.2 μg of an affinity-purified fluorescein isothiocyanate-conjugated rabbit $F(ab')_2$ anti-mouse Fab reagent [Fl-$F(ab')_2\alpha$Fab] which was made against Fab fragments derived from a pool of IgG obtained from a wide variety of mouse strains. This reagent was predominantly reactive with kappa light chains, it has been extensively characterized on the FACS-II[2], and it was the generous gift of Dr. Thomas Chused, National Institutes of Health, Bethesda, Md. The cells were then applied to the FACS-II (Becton, Dickinson & Co., Rutherford, N. J.) for sorting according to a procedure previously described by others (14). Fluorescence-negative cells (50% of the unsorted population) were pooled, washed in the cold, and resuspended for culture. 52% of such cells were latex$^+$ and none of the latex$^+$ (0/300) nor latex$^-$ (0/300) cells were sIg$^+$ when analyzed by fluorescence microscopy with or without restaining with fluorescein isothiocyanate-conjugated rabbit anti-mouse Ig serum.

Culture Conditions. 5×10^5 normal spleen cells or mixtures of 5×10^5 Sephadex G-10-passed spleen cells plus graded numbers of spleen adherent cells were cultured for 4 days in a total volume of 200 μl per flat-bottom well of microtiter plates at 37°C in a 5% CO_2-humidified air atmosphere as previously described (10, 11). Viable cell recovery was generally >50%, and was not significantly affected by the presence or absence of antigen, the passage of spleen cells over Sephadex G-10, or the addition of spleen-adherent cells (10, 11). Two to four parallel cultures were pooled to constitute one culture group; three replicate culture groups were used in all experiments.

Hemolytic PFC Assay. The number of TNP-specific direct PFC was determined for each individual culture group as the difference in number of PFC observed with TNP-conjugated sheep erythrocytes (SRBC) in the absence and presence of TNP-BSA as a specific inhibiting reagent. Greater than 80% of the number of PFC in each responding culture group was inhibited by the concentration of TNP-BSA used (5×10^{-5} M final concentration of TNP, a concentration which does not inhibit anti-SRBC PFC) (10). A variable number of parallel cultures were pooled to constitute one culture group to allow a reasonable number of PFC (>30) to be counted on each slide. Therefore, results were corrected to the number of PFC/10^7 cultured cells, and are expressed as the geometric mean $\times\!\!\!\div$ (standard error) of TNP-inhibitable PFC of triplicate culture groups.

Statistics. Statistical analyses were performed using the two-tailed Student's *t* test on the geometric means $\times\!\!\!\div$ (standard errors) of the uncorrected number of TNP-inhibitable PFC of the culture groups compared, i.e. on the numbers of TNP-inhibitable PFC actually counted in each culture group.

Results

Ability of Spleen Adherent Cells to Function as Accessory Cells in the in Vitro Anti-Hapten PFC Responses of Normal (B10 × B10.A)F_1 Spleen Cells to TNP-(T,G)-A--L, TNP-(H,G)-A--L, and TNP-KLH. To examine the possibility that *Ir* genes regulate the function of accessory cells in the PFC responses to TNP-(T,G)-A--L and TNP-(H,G)-A--L, it was necessary to demonstrate the requirement for such cells in the in vitro responses to these antigens. Normal (B10 × B10.A)F_1 spleen cells responded to TNP-(T,G)-A--L, TNP-(H,G)-A--L, and TNP-KLH (Table I). Passage of these spleen cells over Sephadex G-10 columns markedly reduced the percentage of phagocytic cells, did not alter the percentage of B cells, and somewhat increased the percentage of T cells (Materials and Methods). Sephadex G-10 passage also abrogated the responses to TNP-(T,G)-A--L and TNP-(H,G)-A--L, and significantly diminished ($P < 0.01$) the response to TNP-KLH (Table I). The persistent though significantly diminished response to TNP-KLH was considered to be due to either an accessory cell-independent component of the response to this antigen, or to the residual accessory cells present in the Sephadex G-10-passed population which might have been sufficient to support partial responsiveness to a multi-deter-

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[2] T. M. Chused, S. S. Kassan, and S. O. Sharrow. Increased frequency of cells with low density surface immunoglobulin in NZB mice. Manuscript in preparation.

TABLE I

The in Vitro Anti-Hapten PFC Responses of Normal (B10 × B10.A)F_1 Lymphocytes to TNP-(T,G)-A--L and TNP-(H,G)-A--L are Abolished by Sephadex G-10 Passage and Restored by the Addition of Spleen Adherent Cells

Exp.	(B10 × B10.A)F_1 Spleen cells (5×10^5/culture)	No. of (B10 × B10.A)F_1 spleen adherent cells added per culture	Direct PFC/10^7 cultured cells*			
			No antigen	TNP-(T,G)-A--L	TNP-(H,G)-A--L	TNP-KLH
1	Untreated	0	48(1.76)	752(1.10)	627(1.29)	5754(1.09)
	G-10-Passed	0	0	0	0	1080(1.18)
	G-10-Passed	2.5×10^4	0	772(1.10)	742(1.12)	3741(1.29)
2	Untreated	0	28(1.44)	559(1.02)	317(1.09)	2060(1.10)
	G-10-Passed	0	0	54(1.65)	40(1.49)	1153(1.11)
	G-10-Passed	2.5×10^4	41(1.44)	514(1.10)	275(1.22)	2659(1.18)
	G-10-Passed	2.5×10^4 Depleted of sIg^+ cells‡	0	1148(1.04)	371(1.16)	2418(1.40)

* Geometric mean (SE) of TNP-inhibitable PFC of parallel triplicate culture groups.

‡ Stained for surface Ig with Fl-F$(ab')_2\alpha$Fab and depleted of sIg^+ cells using the FACS-II. Such cells were 52% $latex^+$ and none (0/600) were sIg^+.

minant antigen such as TNP-KLH, but not sufficient to support any response to antigens with more limited numbers of antigenic determinants such as TNP-(T,G)-A--L or TNP-(H,G)-A--L. The responses of G-10-passed spleen cells to all three antigens were reconstituted by the addition of small numbers (2.5×10^4 or 5% of the total cultured cells) of syngeneic (B10 × B10.A)F_1 spleen adherent cells as accessory cells (Table I).

The reconstituting spleen adherent cell populations were prepared by 2-h adherence to glass, treatment with anti-Thy 1.2 serum + C′, irradiation with 1,000 R, and contained three identifiable cell populations: (*a*) 50–80% phagocytic cells, (*b*) 8–15% nonphagocytic sIg^+ cells, and (*c*) 15–25% nonphagocytic, sIg^-, R$\alpha$$MB^-$ cells ("null" cells). To determine whether the nonphagocytic sIg^+ cells were responsible for the accessory cell function of the spleen adherent cell populations, Ig-bearing spleen adherent cells were stained with Fl-F$(ab')_2\alpha$Fab and removed from the other spleen adherent cells using the FACS-II. The remaining cells fully reconstituted the responses to all three antigens tested, even though they contained no detectable sIg^+ cells (Table I, Exp. 2). Thus, a population consisting of sIg^- and radiation-resistant glass-adherent cells, devoid of both T and B cells, enriched in spleen phagocytic cells but also containing nonphagocytic "null" cells was sufficient to reconstitute the responses of Sephadex G-10-passed spleen cells to TNP-(T,G)-A--L, TNP-(H,G)-A--L, and TNP-KLH. It has not yet been determined whether the phagocytic cells or the nonphagocytic "null" cells are responsible for the accessory cell function of the spleen adherent cell populations.

Ability of Spleen Adherent Cells from the Responder, but not Nonresponder, Parent to Restore the Responses of Sephadex G-10-Passed (B10 × B10.A)F_1 Spleen Cells to TNP-(T,G)-A--L and TNP-(H,G)-A--L. It has been previously shown that the in vitro PFC responses of normal spleen cells to TNP-(T,G)-A--L and TNP-(H,G)-A--L are under autosomal dominant, *H-2*-linked *Ir* gene control which map within the *K* or *I-A* regions of *H-2* (11). *H-2^b* strains are responders to TNP-(T,G)-A--L but not TNP-(H,G)-A--L, whereas *H-2^a* strains are responders to TNP-(H,G)-A--L but not TNP-(T,G)-A--L; (*H-2^b* × *H-2^a*)F_1 mice are phenotypically responders to both antigens. The question was then asked

TABLE II

Spleen Adherent Cells from the Nonresponder Parental Strain Do Not Reconstitute the Responses of (B10 × B10.A)F_1 Lymphocytes to TNP-(T,G)-A--L or TNP-(H,G)-A--L

(B10 × B10.A)F_1 Spleen cells (5×10^5/culture)	No. of spleen adherent cells added per culture	Strain of spleen adherent cells	Direct PFC/10^7 cultured cells[*]				
			No antigen	TNP-(T,G)-A--L	P[‡]	TNP-(H,G)-A--L	P
Untreated	0	–	0	452(1.04)	<0.01	348(1.12)	<0.01
G-10-Passed	0	–	41(2.08)	36(1.37)		40(1.49)	
G-10-Passed	2×10^4	(B10 × B10.A)F_1	0	476(1.26)	<0.01	331(1.07)	<0.01
		B10	20(1.0)	337(1.09)	<0.01	41(1.44)	NS
		B10.A	0	60(1.78)	NS	325(1.05)	<0.01
G-10-Passed	1×10^4	(B10 × B10.A)F_1	0	218(1.50)	<0.05	404(1.26)	<0.01
		B10	0	358(1.15)	<0.01	25(1.25)	NS
		B10.A	0	0	NS	193(1.03)	<0.02

[*] Geometric mean (SE) of TNP-inhibitable PFC of parallel triplicate culture groups.

[‡] Compared to responses of G-10-passed spleen cells alone. NS, not significant. ($P > 0.05$).

whether spleen adherent cells from both B10 (*H-2*^{b}) and B10.A(*H-2*^{a}) mice could function as accessory cells in the responses of Sephadex G-10-passed (B10 × B10.A)F_1 spleen cells.

The addition of F_1 spleen adherent cells to Sephadex G-10-passed (B10 × B10.A)F_1 lymphocytes restored the responses to both TNP-(T,G)-A--L and TNP-(H,G)-A--L (Tables I and II). However, the response to TNP-(T,G)-A--L was restored by spleen adherent cells from the responder (B10) parent, but not by such cells from the nonresponder (B10.A) parent (Table II). Similarly, the response to TNP-(H,G)-A--L was restored by spleen adherent cells from the responder (B10.A) parent, but not by such cells from the nonresponder (B10) parent (Table II). Other experiments showed that the ability of spleen adherent cells from responder strains, but not nonresponder strains, to restore these responses was consistent over the entire range of spleen adherent cell doses tested, from 5×10^3 to 1×10^5 per culture (1 to 20% of the total cultured cells). Analogous results were obtained with (A/J × A.BY)F_1 lymphocytes reconstituted with spleen adherent cells from syngeneic F_1, A/J (*H-2*^{a}), or A.BY(*H-2*^{b}) mice (data not shown).

It was concluded that (*a*) spleen adherent cells from the nonresponder parent strain are unable to function as accessory cells in the responses to TNP-(T,G)-A--L or TNP-(H,G)A--L, and that (*b*) the accessory cell function of spleen adherent cells in these responses is controlled by autosomal dominant, *H-2*-linked genes.

The ability of responder and nonresponder parental strain spleen adherent cells to cooperate with G-10-passed F_1 cells was also examined in experiments in which the spleen adherent cells were pulsed with antigen, thoroughly washed, and added as "antigen-presenting" cells without the addition of further antigen. In these experiments, spleen adherent cells from both parental strains presented TNP-KLH to F_1 lymphocytes, but only spleen adherent cells from the responder parent, and not the nonresponder parent, presented TNP-(T,G)-A--L or TNP-(H,G)-A--L (data not shown).

Subregion Mapping of the Genes Controlling the Ability of Spleen Adherent Cells to Restore the Responses of (B10 × B10.A)F_1 Lymphocytes to TNP-(T,G)-A--L and TNP-(H,G)-A--L. To further localize within *H-2* the gene(s) control-

Table III

Spleen Adherent Cells from the Nonresponder Recombinant Strain Do Not Reconstitute and Do Not Suppress the Responses of (B10 × B10.A)F_1 Lymphocytes to TNP-(T,G)-A--L and TNP-(H,G)-A--L

(B10 × B10.A)F_1 Spleen cells (5 × 10^6/culture)	No. of spleen adherent cells added per culture	Strain of spleen adherent cells	Direct PFC/10^7 cultured cells*					
			No antigen	TNP-(T,G)-A--L	P‡	TNP-(H,G)-A--L	P	TNP-KLH
Untreated	0	–	0	495(1.16)	<0.001	322(1.11)	<0.001	3474(1.06)
G-10-Passed	0	–	0	0		0		1143(1.80)
A. G-10-Passed	2 × 10^4	(B10 × B10.A)F_1	0	542(1.19)	<0.001	859(1.30)	<0.001	4650(1.21)
		B10	0	455(1.21)	<0.001	0	NS	4196(1.20)
		B10.A	0	45(1.68)	NS	401(1.34)	<0.01	3238(1.12)
		B10.A(4R)	0	28(1.44)	NS	378(1.06)	<0.001	4277(1.21)
		B10.A(5R)	0	889(1.11)	<0.001	66(2.38)	NS	3301(1.26)
G-10-Passed	1 × 10^4	(B10 × B10.A)F_1	45(1.52)	647(1.68)	<0.01	500(1.12)	<0.001	3424(1.02)
		B10	0	355(1.11)	<0.001	0	NS	2750(1.03)
		B10.A	0	0	NS	347(1.20)	<0.001	2839(1.03)
		B10.A(4R)	0	0	NS	450(1.15)	<0.001	2502(1.28)
		B10.A(5R)	0	375(1.27)	<0.01	0	NS	2739(1.15)
B. G-10-Passed	1 × 10^4 + 1 × 10^4	B10.A(4R) + B10.A(5R)	0	296(1.08)	<0.001	431(1.06)	<0.001	3919(1.09)

* Geometric mean (SE) of TNP-inhibitable PFC of parallel triplicate culture groups.

‡ Compared to responses of G-10-passed spleen cells alone. NS, not significant. ($P > 0.05$).

ling the ability of spleen adherent cells to function as accessory cells in the responses to TNP-(T,G)-A--L or TNP-(H,G)-A--L, spleen adherent cells from the *H-2* recombinant strains B10.A(4R) and B10.A(5R) were added to cultures with G-10-passed (B10 × B10.A)F_1 lymphocytes. The responses of Sephadex G-10-passed F_1 spleen cells to TNP-(T,G)-A--L were restored only by the addition of spleen adherent cells from responder strains (11) (F_1, B10, or B10.A(5R)), but were not restored by the addition of such cells from nonresponder strains (B10.A or B10.A(4R)) (Table III, A). Similarly, the responses of Sephadex G-10-passed F_1 spleen cells to TNP-(H,G)-A--L were restored only by the addition of spleen adherent cells from responder strains (11) (F_1, B10.A or B10.A(4R)), but were not restored by the addition of spleen adherent cells from nonresponder strains (B10 or B10.A(5R)) (Table III, A). Spleen adherent cells from each of these strains fully reconstituted the response of F_1 lymphocytes to TNP-KLH (Table III, A).

Since B10, and B10.A(5R) spleen adherent cells restored the response to TNP-(T,G)-A--L, whereas B10.A and B10.A(4R) adherent cells did not, the gene(s) controlling the accessory function of these cells in the response to TNP-(T,G)-A--L is located within the *K* or *I-A* region of the *H-2*^{b} responder haplotype. Similarly, since B10.A, and B10.A(4R) spleen adherent cells reconstituted the response to TNP-(H,G)-A--L, while B10 and B10.A(5R) adherent cells did not, the gene(s) controlling the accessory function of these cells in the response to TNP-(H,G)-A--L is located within the *K* or *I-A* regions of the *H-2*^{a} responder haplotype. Thus, the autosomal dominant *H-2*-linked genes controlling the accessory function of spleen adherent cells in the responses to TNP-(T,G)-A--L and TNP-(H,G)-A--L are located within the same subregions of *H-2* as the autosomal dominant *Ir* genes controlling the responses of intact spleen cells to these antigens, suggesting that they may be the same genes.

Nonresponder Spleen Adherent Cells Do Not Suppress the Responses of (*B10 × B10.A*)*F_1 Lymphocytes to TNP-*(*T,G*)*-A--L and TNP-*(*H,G*)*-A--L.* The antigen-specific unresponsiveness of F_1 lymphocytes in the presence of spleen adherent cells from nonresponder strains could be due either to an inability of nonresponder spleen adherent cells to function as accessory cells for that antigen or, alternatively, to an active antigen-specific suppression induced by the nonresponder adherent cells. To distinguish between these two possibilities, G-10-passed F_1 lymphocytes were cultured with antigen and with a mixture of B10.A(4R) and B10.A(5R) spleen adherent cells (Table III, B). F_1 lymphocytes cultured with a mixture of spleen adherent cells from both strains, responded to both TNP-(T,G)-A--L and TNP-(H,G)-A--L, in addition to TNP-KLH (Table III, B), demonstrating that the responses to these antigens were not actively suppressed by the presence of nonresponder adherent cells.

Discussion

The present study demonstrates that the accessory function of non-T, non-B spleen adherent cells in the in vitro primary IgM anti-hapten PFC responses to the synthetic antigens TNP-(T,G)-A--L and TNP-(H,G)-A--L is regulated by autosomal dominant genes located in the *K* or *I-A* regions of the *H-2* complex. In the present in vitro experiments, unprimed ($H\text{-}2^b$ × $H\text{-}2^a$)F_1 spleen cells passed over Sephadex G-10 columns were unable to respond to TNP-(T,G)-A--L or TNP-(H,G)-A--L. The responses to these antigens were reconstituted by the addition of spleen adherent cells from the responder parental strain, but not from the nonresponder parental strain. The ability of spleen adherent cells to reconstitute these responses was shown to be antigen- and strain-specific in that: (*a*) spleen adherent cells from each parental strain reciprocally reconstituted the response to one synthetic antigen but not the other, and (*b*) spleen adherent cells from both parental strains reconstituted the response to TNP-KLH. Furthermore, experiments which utilized spleen adherent cells from parental *H-2* recombinant strains demonstrated that the ability of these cells to function as accessory cells in these responses was regulated by autosomal dominant, *H-2*-linked genes located in the *K* or *I-A* regions of the responder *H-2* complex. Since the *Ir* genes controlling responsiveness in vitro to TNP-(T,G)-A--L and TNP-(H,G)-A--L and those controlling responsiveness in vivo to (T,G)-A--L and (H,G)-A--L are also located in the *K* or *I-A* regions of the responder *H-2* complex, this result suggests that these *Ir* genes and the genes controlling accessory cell function in these responses may be the same.

The inability of spleen adherent cells from nonresponder strains to function as accessory cells in these responses cannot be due to histocompatibility barriers to cooperation between adherent cells and lymphocytes since (responder × nonresponder)F_1 lymphocytes were reconstituted with semi-syngeneic spleen adherent cells which restored the responses to both TNP-KLH and to at least one of the synthetic antigens. The possibility of antigen-nonspecific allogeneic effects was minimized by not using allogeneic spleen adherent cells and by always depleting the spleen adherent cell populations of T cells and irradiating them. Moreover, allogeneic effects cannot account for the present data since the results were specific for both antigen and the *H-2* type of the spleen adherent cells. The inability of spleen adherent cells from nonresponder strains to reconstitute the responses of one of the synthetic antigens was also not due to antigen-specific suppression induced by these cells, since the mixing of spleen adherent cells from both responder and nonresponder strains did not suppress the responses to any of the antigens studied.

The present results are consistent with those reported in secondary guinea pig T-cell proliferation assays (15, 16) in which peritoneal exudate cells from the responder but not nonresponder parental strain effectively "presented" antigen to in vivo primed F_1 T cells. The present data extend these previous observations in several ways. First, these data demonstrate that accessory cells can participate in *Ir* gene regulation in antibody-forming cell responses. Second, since the PFC responses describod in this report were generated by unprimed F_1 spleen cells, the observed results cannot be due to any effect of previously priming these cells in vivo in the presence of F_1 macrophages. The ability to avoid the potential restrictions on cell-cell collaboration induced by previously antigen-priming the responding lymphocytes is one of the advantages of examining an *Ir* gene-controlled primary IgM response. Third, as noted above, the genes controlling the accessory cell function of spleen adherent cells have been localized to the same region(s) of the *H-2* complex as the *Ir* genes which control in vitro and in vivo responsiveness to the same antigens and, hence, may be the same genes.

The present results do not conflict with previous reports that T cells or B cells express *Ir* genes in responses to (T,G)-A--L or (H,G)-A--L (4–9), since the present study does not examine the possibility that T cells or B cells also express *Ir* genes, and since these previous studies did not exclude the possibility that accessory cells express *Ir* genes. However, *Ir* gene control of accessory cell function could have accounted for these previous results. The present results do appear to conflict with those reported for the *Ir* gene-controlled primary IgG response to the antigen $Glu^{60}Ala^{30}Tyr^{10}$ (17, 18). The reasons for this discrepancy are unclear, but they may relate to the possibility that *Ir* gene defects for different antigens or different strains are expressed in different cell types. Studies to determine the factors actually responsible for these disparate results are in progress.

Several mechanisms have been proposed for the participation of accessory cells in *Ir* gene regulation (15, 16). *Ir* gene function could be inherent in accessory cells per se, and expressed as the ability of these cells to present antigenic determinants in an appropriate fashion for competent T cells or B cells (16). Alternatively, in order to maintain the more traditional concepts that *Ir* genes function in T or B lymphocytes, it can be hypothesized that *Ir* genes regulate the interaction of accessory cells with T or B lymphocytes (15). Regardless of which hypothesis is correct, the present data demonstrate that the localization to *H-2* of the genes controlling responsiveness to TNP-(T,G)-A--L and TNP-(H,G)-A--L could be solely due to the *H-2* region genes of the accessory cells. Consequently, it may not be necessary for lymphocyte receptors for antigen to themselves be *H-2*-region products in order to explain the linkage to *H-2* of the genes controlling the responses to these antigens.

The most straightforward interpretation of the data presented in this report is that the *H-2*-linked genes controlling the accessory cell function of spleen adherent cells in responses to TNP-(T,G)-A--L and TNP-(H,G)-A--L are the same as the *H-2*-linked *Ir* genes controlling in vitro and in vivo responsiveness to these antigens. These results do not exclude the possible expression of *Ir* genes by T or B lymphocytes as well.

Summary

The possibility was investigated that *Ir* genes regulate the function of cells other than T or B cells in the primary IgM responses to the synthetic antigens trinitrophenylated poly-L-(Tyr,Glu)-poly-D,L-Ala--poly-L-Lys [TNP-(T,G)-A--L] and trinitrophenylated poly-L-(His,Glu)-poly-D,L-Ala--poly-L-Lys [TNP-(H,G)-A--L]. The primary responses of (B10 × B10.A)F_1 spleen cells to both antigens were abrogated by Sephadex G-10 passage, and restored by the addition of spleen adherent cells. The cell type in the spleen adherent cell population active in reconstituting the responses to TNP-(T,G)-A--L and TNP-(H,G)-A--L was a non-T, non-B, radiation-resistant, glass-adherent spleen cell. The responses of Sephadex G-10-passed (responder × nonresponder)F_1 spleen cells to TNP-(T,G)-A--L or TNP-(H,G)-A--L were reconstituted by spleen adherent cells from only responder strains. Spleen adherent cells from F_1 mice reconstituted the responses to both antigens. Spleen adherent cells from each of the strains tested reconstituted the non-*Ir* gene-controlled response to a third antigen, TNP-keyhole limpet hemocyanin. The inability of spleen adherent cells from nonresponder strains to reconstitute the responses to either TNP-(T,G)-A--L or TNP-(H,G)-A--L was not a result of active suppression induced by the presence of nonresponder adherent cells, since a mixture of responder and nonresponder spleen adherent cells reconstituted the responses to both antigens. The use of spleen adherent cells from recombinant strains demonstrated that the autosomal dominant genes controlling the ability of spleen adherent cells to function as accessory cells in the responses to TNP-(T,G)-A--L and TNP-(H,G)-A--L are located in the *K* or *I-A* regions of the responder *H-2* complex, the same region(s) of *H-2* as the *Ir* genes controlling overall in vitro and in vivo responsiveness to these antigens.

The authors particularly wish to thank Dr. Gene Shearer for his encouragement and suggestions throughout the course of this work; Dr. Edna Mozes for the generous gift of (H,G)-A--L; Dr. Thomas Chused for the gift of the Fl-F(ab')$_2$αFab reagent; Ms. Susan Sharrow for her expert operation of the FACS-II; Doctors William Terry, David Sachs, Alan Rosenthal, William Paul, Dinah Singer, and David Pisetsky for critically reviewing this manuscript; Mr. Walter Lyles and Mr. Francis Jones for their care of experimental animals; Mr. John Williams for his expert technical assistance; and Ms. Judy Steckel for the preparation of the manuscript.

Received for publication 14 November 1977.

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IN VITRO INDUCTION OF CYTOTOXIC EFFECTOR CELLS WITH SPONTANEOUS KILLER CELL SPECIFICITY

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Recent cytotoxicity studies in humans have shown that normal individuals have a defined lymphocyte subpopulation which is spontaneously cytotoxic to in vitro propagated cell lines (1-4). This phenomenon we have earlier called spontaneous lymphocyte-mediated cytotoxicity and the involved effector cell spontaneous killer (SK)[1] cell (1). In the mouse a similar phenomenon has been designated natural cytotoxicity (5). The human SK cell has been shown to express receptors for IgG (Fc receptors) and partly receptors for split products of the third complement factor (C3 receptors), whereas they do not express surface bound immunoglobulin (1, 4, 6). Whether the SK cells express receptors for sheep erythrocytes (RBC), a human T-lymphocyte marker, is a more controversial question. In most reports most SK activity is to be found in the non-sheep RBC binding cell fraction (1-3) whereas West et al. have demonstrated that SK cells can be made to bind sheep RBC under certain optimum conditions (4). Thus, from available information one can conclude that SK cells have Fc receptors and that they may or may not express sheep RBC receptors and that they are, so far, indistinguishable, by various fractionation procedures, from effector cells active in antibody dependent cellular cytotoxicity (7).

The biological significance of spontaneous cytotoxicity is, at present, unclear. According to the bias of the investigator, it may be conceived as anything ranging from an in vitro artifact to an important system in the regulation of the immune response or in the protection against tumor development (3). Beside the actual in vivo role SK cells may play, it is also unclear how they are generated, what type of target cell antigens they recognize, and by what mechanism they kill the target cells. In the present paper we have approached the questions of SK cell recognition and SK cell activation.

With regard to target cell recognition, we as well as others have found that SK cells from different individuals seem to have a similar target cell specificity as they consistently kill some cell lines better than others. Recently we have been able to extend these studies to show that SK cells preferentially kill cell lines that are derived from leukemic tumor cells as compared to cell lines derived from normal lymphocytes (8 and Table II). In the present work we have taken advantage of this relative target cell specificity to demonstrate that killer cells with a similar specificity may actually be generated in vitro. We have thus found that stimulation of peripheral lymphocytes with autologous or allogeneic B-cell lines for a 5-day period leads to a strong induction of

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[*] Supported by National Institute of Health grant CA12800, Swedish Cancer Society, and Cancer Research Institute, Inc.

[‡] Supported by U. S. Public Health Service grant T32-AI07126.

[1] *Abbreviations used in this paper*: ADCC, Con A, concanavalin A; LDCC, lectin dependent cellular cytotoxicity; MLC, mixed lymphocyte culture; PBS, phosphate-buffered saline; RBC, erythrocytes; RSI, relative specificity index; SK, spontaneous killer; SRBC, sheep erythrocytes.

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cytotoxic cells and that these effector cells kill the same target cells as those which are susceptible to SK cell cytotoxicity. We find this interesting as it may reflect how SK cells are generated in vivo as autologous lines induce cytotoxicity as well as allogeneic lines. If so, that would further define the nature of the SK system as several earlier reports have dealt with the mechanism and characteristics of B-cell line stimulation (9–13).

Materials and Methods

Purification of Lymphocytes. Lymphocytes were purified by centrifugation of heparinized whole blood on Ficoll-Hypaque gradients (14). Phagocytic cells were removed by the iron carbonyl powder technique (14).

Maintenance of Cell Lines. Cell lines were maintained as suspension cultures in RPMI-1640 medium supplemented with antibiotics and 10% fetal calf serum. Cell cultures were fed twice weekly.

Stimulation with B-Cell Lines. Stimulation cultures were set up in upright Corning 25 cm^2 tissue culture flasks (no. 25100). Responding lymphocytes were at a concentration of 1×10^6/ml with a responder:stimulator ratio of 2:1 or 3:1. Cultures were kept for 5 days at tissue culture conditions, whereafter the number of viable cells was calculated by trypan blue exclusion and the amount of DNA synthesis assayed by [^{3}H]thymidine incorporation in 0.1 ml of cultures (see below). In case of poor viability on the part of the responding cells, these were run on Ficoll-Hypaque gradients to remove dead cells and cell debris. The stimulating B-cell lines were established from normal individuals by Doctors C. Spina and M. Jobin at the Department of Microbiology and Immunology, University of California at Los Angeles, and were mitomycin C treated at a concentration of 50 μg/ml for 20–30 min at 37°C and washed three times before used. Cultures were set up in RPMI-1640 medium with 20% heat-inactivated autologous serum. Unstimulated control lymphocytes were tissue cultured for the 5-day period at a higher cell concentration of 2–4 $\times 10^6$/ml as those cells otherwise would show poor viability on day 5.

Cytotoxicity Tests. Cytotoxicity tests were performed by ^{51}Cr release in standard V-shaped microplates (Cooke Laboratory Products Div., Dynatch Laboratories, Inc., Alexandria, Va., no. 1-220-25A) in a total vol of 150 μl RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum. Target cells were labeled with ^{51}Cr (New England Nuclear, Boston, Mass.) at a concentration of 200 μCi/5 $\times 10^6$ target cells for 45 min at 37°C and washed three times. 10,000 target cells were used for each microplate well from which 50 μl supernate was harvested after an incubation period of 3-5 h and cytotoxicity estimated by the amount of released radioactivity. Cytotoxicity was calculated according to the standard formula:

$$\frac{\text{test release} - \text{spontaneous release}}{80\% \text{ of total label} - \text{spontaneous release}} \times 100.$$

Lectin dependent cellular cytotoxicity was also performed in V-shaped microplates as described for spontaneous cytotoxicity above. Concanavalin A (Pharmacia, Uppsala, Sweden) was used at a final concentration of 2 μg/ml which earlier had been found optimal in inducing DNA synthesis as well as lectin dependent cellular cytotoxicity (LDCC).

Calculation of Relative Specificity Index (RSI). To establish the specificity of various stimulated and unstimulated lymphocyte populations, we established an RSI. This index is based on the finding that certain cell lines are more susceptible to spontaneous cytotoxicity than others (Table I). We have picked Molt-4 as representative for susceptible target cell and Raji as representative for resistant target cells. Raji is thus an exception to the rule that tumor-derived targets in general are highly susceptible to SK cell cytotoxicity as can be seen in Table I. In calculating the index as described in Fig. 1, we establish an aggressor:target ratio dependent cytotoxicity curve for the Molt-4 and determine the amount of cytotoxicity at the early slope of this curve and the Raji cytotoxicity at the same aggressor to target ratio. At this point we estimate Molt-4 cytotoxicity and Raji cytotoxicity in a situation where there is an excess of aggressor cells. RSI is then calculated as Molt-4 cytotoxicity minus Raji cytotoxicity divided by Molt-4 cytotoxicity. In case Raji killing is higher than Molt-4 killing, this is indicated by a minus sign before the index. With equal killing of Molt-4 and Raji, the index will thus be 0.0 with only Molt-4 killing 1.0 and with 50% Raji killing, as compared to Molt-4 killing, 0.5. In calculation of RSI, no Molt-4 cytotoxicity values below 30% are accepted as these seldom represent slope values.

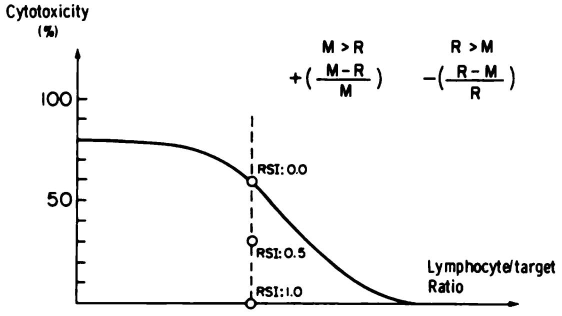

FIG. 1. For explanation of the relative specificity index, see Materials and Methods.

Assay for DNA Synthesis by Uptake of [^{3}H]Thymidine. Of the stimulated cultures, 0.1 ml was harvested in triplicates in standard microplates and 2 μCi [^{3}H]thymidine put into each well. After an incubation period of 3–4 h, the microplates were frozen down to be eventually harvested in an automated multiple sample harvester and incorporated radioactivity measured by counting each sample in 5 ml of Econofluor (New England Nuclear).

Fractionation of Sheep RBC Binding Lymphocytes. Purified lymphocytes were mixed with sheep RBC at a lymphocyte: sheep erythrocyte (SRBC) ratio of 50:1 in 20% fetal calf serum. This cell mixture was then spun down in 50 ml round-bottomed centrifuge tubes and incubated for 15 min at 37°C and subsequently for 1 h at 4°C. Thereafter, the cells were gently resuspended and spun at 4°C on cold Ficoll-Hypaque. By repeating this procedure twice, non-sheep RBC binding lymphocytes, which were harvested from the interface, contained less than 5% of contaminating rosette-forming cells.

Fractionation of Fc Receptor-Bearing Lymphocytes. Fc receptor-bearing lymphocytes were fractionated with a monolayer immune complex fractionation technique as described elsewhere (15). Briefly, human IgG was dissolved in phosphate-buffered saline (PBS)-A (without divalent cations) to a final concentration of 0.5 mg/ml. 3 ml of this solution was poured into 60 × 15 mm tissue culture dishes (BioQuest, BBL, & Falcon Products, Becton, Dickinson & Co., Cockeysville, Md. no. 3002) and incubated therein for 30 min at room temperature and for 30 min at 4°C. Thereafter, the dish was washed three times with 2 ml of PBS-A and subsequently filled with 2 ml of a rabbit anti-human IgG serum. The antiserum was used at a final concentration of 1:20 and the dish was incubated for 30 min at room temperature for an additional 30 min at 4°C and then washed three times with PBS-A. Thereafter, purified lymphocytes, suspended in PBS-A with 10% fetal calf serum at a concentration of 5–7.5 × 10^6/ml, were added in a total vol of 2 ml to the plates which were then spun at 500 rpm for 5 min, turned 180°C, and spun for another 5 min. After the last centrifugation, the Fc receptor-bearing cells adhered to the plastic surface, and unbound cells could be collected by three washes with 2 ml PBS-A. Fc receptor positive cells could be partially collected accordingly: 2 ml of a protein A solution (Pharmacia, Uppsala, Sweden) at a concentration of 30 μg/ml was poured into each dish which then was incubated with slow rocking at 37°C for 2 h. During this time period, protein A competed with Fc receptors for binding sites on the IgG molecule, and part of the cell detached from the immune complex monolayers. The recovery of Fc receptor negative cells was approximately 71%, and of the Fc receptor positive cells approximately 14% of the initial cell number. Of plate-bound cells, approximately 36% could be eluted with protein A.

Results

In Vitro Parameters Influencing SK Cytotoxicity. It is well-known that many factors in the in vitro environment may influence SK cytotoxicity. It has thus already been shown that ammonium chloride, when used for RBC lysis, may decrease cytotoxicity as may preincubation of effector cells at 4°C or freezing of effector cells (16). Furthermore, we have found that autologous or heterologous RBC may increase cytotoxicity (M. Jondal, unpublished observa-

TABLE I

[illegible] K-562

[illegible]	[illegible]	[illegible]	[illegible]								
			[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	Con-trol
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

[illegible] with an enzyme concentration of 10 µg/ml. [illegible] for spontaneous cytotoxicity with or [illegible] serum concentrations starting from a final concentration of 1:3 [illegible] Control test medium consisted of RPMI-1640 with 10% [illegible]

[illegible] that the serum source used in a 5-day culture may either increase or decrease SK cytotoxicity. We have made the following observations about the serum source. Fetal calf serum can sometimes act as a mitogen and can thereby increase cytotoxicity, pooled human AB+ serum is inhibitory and little SK killing can be detected after 5 days, autologous serum can either be inhibitory or have no influence in the system. Table I exemplifies the effect of autologous serum on the SK cytotoxicity against the cell line K-562. One can see a slight increase of killing with high serum concentrations at time 0 whereas the same serum concentration is inhibitory at 24 h, but an increase of cytotoxicity can be noted at higher serum dilutions at this time point. This serum effect does not require serum present during the actual cytotoxicity test and is equally detectable on trypsinized lymphocytes. In our further tissue culture work involving culture stimulated lymphocytes and control lymphocytes, we chose to work with [illegible] autologous serum but it should be remembered that this serum concentration often decreases NK cytotoxicity of unstimulated control lymphocytes. In Table III this effect can be seen, with decrease of control cytotoxicity with donors 1, 6, and 7 whereas donor 8 remains virtually unchanged.

Target Cell Specificity in the SK System. Table II summarizes the susceptibility of various target cells to spontaneous cytotoxicity as reported in more detail elsewhere (8). It can thus be seen that all of the tumor-derived target cells except Raji were classified as susceptible whereas all of the target cells derived from normal lymphocytes were classified as resistant. We defined resistance as equal to or less than 12% mean specific release in several repeat experiments during a 3.5 h ^{51}Cr release assay at a lymphocyte target ratio not higher than 50:1. The specificity as presently defined is thus of a relative nature as by altering the technical conditions such as by increasing the testing time or by increasing the number of effector cells some of the resistant lines will give a higher specific release than 12%. To further work with this relative specificity

TABLE II

Cell Lines Derived from Normal Lymphocytes are Less Susceptible to Spontaneous Cytotoxicity Than Most Cell Lines Derived from Leukemic Tumor Cells

Origin of cell line	Name of cell line	Number of cell lines tested	Susceptible/ resistant to spontaneous cytotoxicity
Burkitt's lymphoma (17)	P3HR-1	1	1/1
Burkitt's lymphoma (18)	BJAB	1	1/1
Burkitt's lymphoma (19)	Daudi	1	1/1
Burkitt's lymphoma (20)	Raji	1	0/1
Acute lymphocytic leukemia (21)	Molt-4	1	1/1
Acute lymphocytic leukemia (22)	HSB-II	1	1/1
Chronic myeloid leukemia (23)	K-562	1	1/1
Normal lymphocytes	See legend	15	0/15

Cell lines were tested for susceptibility to spontaneous cytotoxicity in a short-term (3-5 h)^{51}Cr release assay. Susceptibility was defined in the present context as a higher specific mean release than 12%, in several repeat experiments, at a lymphocyte:target ratio not higher than 20:1. Cell lines derived from normal lymphocytes were either spontaneously transformed from Epstein-Barr virus seropositive individuals or transformed by the in vitro addition of virus by Doctors Celsa Spina and M. Jobin at the Department of Microbiology and Immunology, University of California at Los Angeles, and by Dr. Richard Gatti at the Department of Pediatric Oncology, Cedars-Sinai Medical Center, Los Angeles.

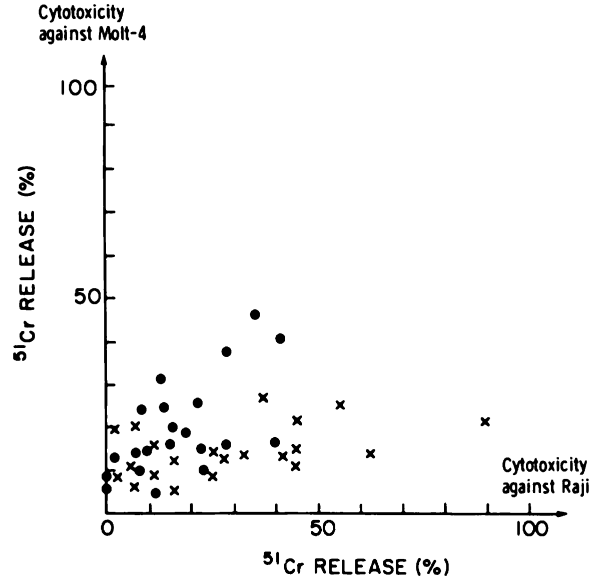

FIG. 2. Unfractionated lymphocytes or lymphocytes depleted for Fc and complement receptor positive cells by the EAC(7S) rosette sedimentation procedure (24) were tested for specific Con A dependent cellular cytotoxicity as described in Materials and Methods. ×, unfractionated, ●; Fc receptor negative.

of the SK system, we selected one line as representative for the susceptible group (Molt-4) and one line as representative for the resistant group (Raji) and defined an RSI as described in the Materials and Methods section. In Fig. 3 it can be seen that there is a tendency towards lesser specificity, i.e., more Raji

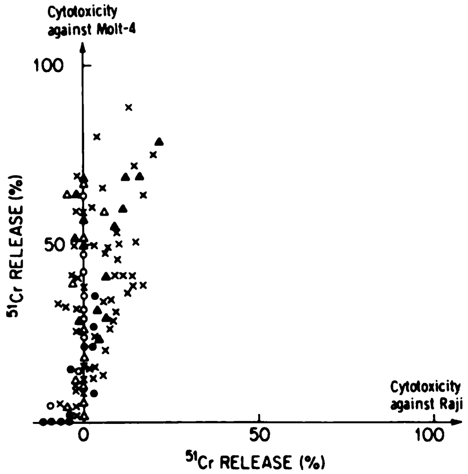

FIG. 3. Different unstimulated lymphocyte subpopulations fractionated as described in Materials and Methods were tested for spontaneous cytotoxicity against Molt-4 and Raji. ×, unfractionated; △, E receptor positive; ○, E receptor negative; ▲, Fc receptor positive; ●, Fc receptor negative (20 experiments).

killing with increasing Molt-4 cytotoxicity and that this seems to occur with all tested lymphocyte subpopulations. In later experiments with SRBC fractionated cells, we have seen that this also is true for non-SRBC binding lymphocytes although these cells appear as comparatively more Molt-4 specific in Fig. 3.

In Table VII we have calculated the RSI from 12 experiments with unfractionated lymphocytes and found a mean RSI of 0.84.

To exclude that the relative resistance on part of the Raji cell line was due to a general resistance to cell-mediated lysis, we tested the susceptibility of Raji cells and Molt-4 cells to concanavalin-A (Con-A) dependent cytotoxicity. From Fig. 2 in which we have plotted specific Con-A dependent Raji killing against specific Con-A dependent Molt-4 killing, it is clear that Raji cells are as susceptible to lysis as Molt-4 cells. With unfractionated cells there is relatively less Con A dependent cytotoxicity against Molt-4 as compared to killing with Fc receptor depleted cells which is related to the fact that there is a high SK background with the Molt-4 cell line. The results in Fig. 2 are furthermore in line with our earlier results showing that SK resistant target cells can be killed by LDCC and that relatively little LDCC can be detected against target cells that are very susceptible to spontaneous cytotoxicity and especially so with isolated Fc receptor positive lymphocytes (25).

In summary, these results with unstimulated "true" SK cells show that these cells do have a relative specificity which may be tumor related and that the specificity most probably is related to lack of recognition at the effector cell level as the resistant target cells become susceptible in the presence of Con A and finally that the RSI of SK cells when defined by killing against Molt-4 versus killing against Raji is 0.84.

Induction of Cytotoxicity and DNA Synthesis by Stimulation with B-Cell Lines. Stimulation of peripheral lymphocytes with mitomycin C-treated allogeneic or autologous B-cell lines result in increased DNA synthesis and induction of cytotoxic effector cells. In Table III a representative experiment is

TABLE III

Cytotoxicity against MOLT-4 by Spontaneous Killer Cells and by Killer Cells Induced by Stimulation of Unfractionated Lymphocytes with an Allogeneic B-Cell Line

Exp.	Donor	Unstimulated					Stimulated			
		Day 0		Day 5			Day 5			DNA Synthesis
		20:1	10:1	20:1	10:1	5:1	20:1	10:1	5:1	
		^{51}Cr release								*cpm/0.1 ml culture*
39	Macintosh	20	12	2	1	0	72	74	64	55,082 (194)
	Myers	51	41	ND			75	56	49	39,166 (ND)
	Roman	20	13	ND			68	40	31	66,820 (ND)
	Hopkins	37	26	ND			82	75	80	93,251 (ND)
	Luthardt	63	47	ND			87	83	89	82,587 (ND)
	Kechejian	35	22	22	9	5	81	78	79	66,136 (1,339)
	Flink	31	29	14	3	2	82	81	76	96,065 (658)
	Jondal	46	41	61	46	28	83	90	79	90,845 (2,964)

Lymphocytes were tested for spontaneous cytotoxicity against Molt-4 at days 0 and 5 and after stimulation for 5 days with an allogeneic B-cell line. Cell cultures were set up as described in Materials and Methods and DNA synthesis estimated from 0.1 ml of total cell culture, control values without stimulation are given in parentheses.

TABLE IV

Generation of Killer Cells with SK Specificity during Stimulation of Unfractionated Lymphocytes with an Autologous and an Allogeneic B-Cell Line

Exp.	Donor	Stimulator	Cytotoxicity against Molt-4			Cytotoxicity against Raji		
			40:1	20:1	10:1	40:1	20:1	10:1
			^{51}Cr Release					
A	Fahey	None	19	9	2	−2	−3	−1
		Autologous line	72	65	62	5	4	1
		Allogeneic line	85	84	68	8	6	3

Lymphocytes were stimulated with an autologous and an allogeneic B-cell line for 5 days and tested for cytotoxicity against the cell lines Molt-4 and Raji.

given in which eight different lymphocyte donors were stimulated with the same allogeneic B-cell line and subsequently tested for [^{3}H]thymidine incorporation and cytotoxicity against Molt-4. With each donor there is a strong proliferative response and with all donors there is a marked increase in the capacity of the cells to kill Molt-4 except possibly with stimulated lymphocytes from donor 2.

In Table IV we have compared the capacity of allogeneic vs. autologous B-cell lines to generate Molt-4 killing. It is thus clear that generation of killer cells is not related to an expression of non-self HLA. In Table VII, experiment A and 30 and in Table VIII, experiment B, are further support of this conclusion. This finding is in line with earlier reports which also show that autologous B-cell lines act as strong stimulators in this system (9, 13).

The property to induce DNA synthesis and killer cells is mainly confined to B-cell lines (26). This is illustrated in Table V in which a slight increase of

TABLE V

Preferential Induction of DNA Synthesis and Generation of Killer Cells with SK Specificity during Stimulation of Unfractionated Lymphocytes with B-Cell Lines as Compared to Stimulation with T-Cell Lines

Exp.	Donor	Stimulator	Cytotoxicity against Molt-4			Cytotoxicity against Raji			DNA Synthesis
			12:1	6:1	3:1	12:1	6:1	3:1	
			^{51}Cr release						*cpm/0.1 ml culture*
16	Elwell	None	15	4	−2	−2	−1	0	1,121
		Molt-4 (T)	25	15	4	−3	−3	0	2,622
		HSB-II (T)	31	21	12	−3	−1	−4	3,187
		322 (B)	74	67	59	17	2	−3	19,759
		327 (B)	60	50	46	−3	2	−1	10,998

Lymphocytes were stimulated with two allogeneic T-cell lines, Molt-4 and HSB-II, and with two allogeneic B-cell lines, 322 and 327, and tested for cytotoxicity against Molt-4 and Raji and for [^{3}H]thymidine incorporation.

TABLE VI

Comparison between the Capacity of Allogeneic Lymphocytes Versus Autologous B Line Cells to Stimulate Generation of Killer Cells with SK Specificity from Lymphocytes Initially Depleted of SK Cells

Exp.	Donor	Stimulator	Cytotoxicity against Molt-4			Cytotoxicity against Raji		
			20:1	10:1	5:1	20:1	10:1	5:1
			^{51}Cr release					
B	M. Brown	Allogeneic lymphocytes	25	13	5	14	6	−1
		Autologous B-cell line	69	62	51	11	5	4
		None (unfractionated cells at day 0)	32	26	19			
		None (depleted cells at day 0)	3	0	1			

Lymphocytes initially depleted for SK cells by EAC (7S) rosette sedimentation (24) were stimulated by fresh allogeneic lymphocytes and by autologous B-cell line cells for 5 days and tested for cytotoxicity against Molt-4 and Raji cells. The proliferative response was strong in both instances as estimated by the percentage of blast transformed responder cells.

[^{3}H]thymidine incorporation and Molt-4 cytotoxicity occurs with the two T-cell lines, Molt-4 and HSB-II, whereas the B-cell lines 322 and 327 trigger a much stronger response. It may thus be tentatively concluded that the difference between B- and T-cell lines to act as stimulators is more of a quantitative than of a qualitative nature.

We then investigated whether lymphoproliferation induced by fresh allogeneic lymphocytes in a conventional mixed lymphocyte culture (MLC) would result in a generation of killer cells with SK specificity. A representative experiment is shown in Table VI from which it can be concluded that much less SK cytotoxicity is generated by allogeneic lymphocytes as compared to autologous B-cell lines. Furthermore, the MLC generated killer cells have a much lower Molt-4 specificity with an RSI of 0.44 at a lymphocyte:target ratio of 20:1 (although the Molt-4 cytotoxicity is too low to allow a correct RSI calculation

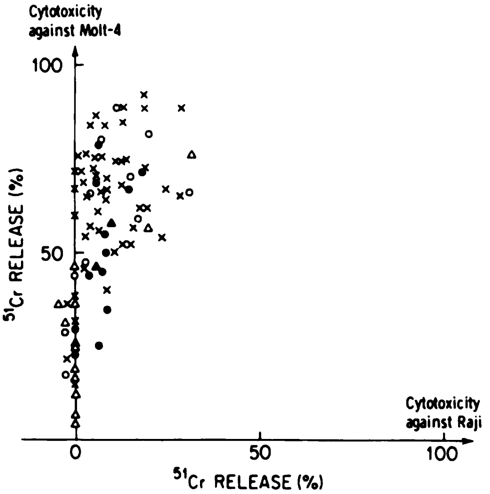

FIG. 4. Different stimulated lymphocyte subpopulations fractionated as described in Materials and Methods were tested for spontaneous cytotoxicity against Molt-4 and Raji ×, unfractionated; △, E receptor positive; ○, E receptor negative; ▲, Fc receptor positive; ●, Fc receptor negative (17 experiments).

[Materials and Methods]) compared to an RSI of 0.88 at a lymphocyte:target ratio of 10:1 for killer cells generated by the autologous B-cell line. In this experiment, killer cells were generated from virtually noncytotoxic lymphocyte populations depleted for EAC (7S) receptor-bearing cells. No test for [^{3}H]thymidine incorporation was done in this experiment, but morphologically, by making a rough estimate of the number of responding blast cells, it was clear that both the allogeneic fresh lymphocytes and the autologous B-cell lines caused a strong proliferative response.

In Fig. 4 we have summarized 17 experiments in which unfractionated, E receptor positive or E receptor negative lymphocytes or Fc receptor positive or Fc receptor negative lymphocyte subpopulations were stimulated with B-cell lines and subsequently tested for Molt-4 and Raji cytotoxicity. The purpose of Fig. 4 was to get a quantitative relationship between the Molt-4 killing and Raji killing and to compare the specificity of generated killer cells to that of unstimulated SK cells. Fig. 4 resembles essentially Fig. 3 in that the Molt-4 specificity is high below 50% of cytotoxicity and has a tendency to become less specific above 50%, and in that killer cells with a similar specificity can be generated from all cell populations, even from initially noncytotoxic Fc receptor negative lymphocytes.

The RSI of B-cell line generated killer cells from unfractionated lymphocytes have been calculated in Table VII. The mean RSI from 18 experiments was found to be 0.86 as compared to a mean RSI of unstimulated lymphocytes of 0.84 (12 experiments). It is thus clear that these two different categories of cytotoxic effector cells are very similar as far as target cell specificity goes. In Table VIII a similar RSI calculation has been done with killer cells generated from mostly noncytotoxic Fc receptor negative lymphocyte populations (except in experiments 27, 29, and 37). In seven experiments the killer cells had a mean RSI of 0.85 which means that these killer cells also have a very similar specificity to

TABLE VII

RSI and Amount Generated Cytotoxicity against MOLT-4 during Stimulation of Unfractionated Lymphocytes with B-Cell Lines

Exp.	Donor	Unstimulated		RSI	Ratio	Stimulated		Generated cytotoxicity
		Ratio	Cytotoxicity			Cytotoxicity	RSI	
			% ^{51}Cr release			*% ^{51}Cr release*		*% of total*
A	Fahey	40:1	19	?	10:1	68	0.96	97
					20:1(AS)	65	0.94	86
15	Folkert	10:1	70	0.99	10:1	75	0.99	7
16	Elwell	25:1	33	0.82	25:1	71	1.00	54
					25:1	73	0.77	77
27	Rodmar	15:1	72	0.97	3:1	85	0.85	35
28	D. Brown	40:1	35	1.00	5:1	61	1.00	93
30	Purdue	30:1	40	0.86	15:1(AS)	85	0.92	74
					7:1	86	0.85	88
33	Stein	40:1	37	0.89	10:1	68	0.96	81
35	Vesera	20:1	12	?	5:1	67	0.90	91
37	Gunnels	6:1	50	0.60	25:1	36	0.69	-100
37	Birgitta	25:1	17	?	12:1	68	0.75	84
38	Fehniger	10:1	72	0.79	5:1	62	0.69	6
38	Shope	20:1	10	?	10:1	51	0.69	93
39	Macintosh	20:1	20	?	5:1	64	0.89	100
39	Kechejian	20:1	35	0.77	5:1	79	>0.76	94
39	Flink	40:1	34	0.72	5:1	86	>0.86	98
39	Jondal	20:1	46	0.81	5:1	79	>0.69	65
42	Thiele	25:1	15	?	6:1	56	0.77	93
43	Karjala	30:1	38	0.92	15:1	71	0.92	72
				Mean: 0.84(12)			Mean: 0.86(18)	

Amount induced Molt-4 cytotoxicity is calculated as: cytotoxicity with stimulated cells minus cytotoxicity with unstimulated cells through cytotoxicity with stimulated cells and estimated by the lymphocyte:target ratio given for the stimulated cells. The relative specificity index was calculated as described in Materials and Methods.

that of SK cells. The lymphocyte donors which had unstimulated Fc receptor negative killer cells in experiments 27, 29, and 37 were subsequently retested at two separate occasions. At both times all three donors were found to have Fc negative effector cells although the amount of cytotoxicity expressed in this subpopulation, as compared to unfractionated cells, varied.

Discussion

In the present work we have taken advantage of the fact that human SK cells have a clearly defined specificity when assayed in a short-term ^{51}Cr release assay as they preferentially recognize and kill target cells of tumor origin (8 and Table I). Furthermore, we have demonstrated that this specificity is not due to a nonspecific resistance to cell-mediated lysis on the part of the SK resistant target cells as these cells were killed when Con A was present in test medium. Con A does presumably in this situation only act by bringing effector and target cells together and can thus be considered as a substitute for target

TABLE VIII

RSI and Amount Generated Cytotoxicity against MOLT-4 during Stimulation of Lymphocytes Depleted for Fc Receptor-Bearing Cells with B-Cell Lines

Exp.	Donor	Unstimulated		RSI	Ratio	Stimulated		Generated cytotoxicity
		Ratio	Cytotoxicity			Cytotoxicity	RSI	
			% ^{51}Cr release			*% ^{51}Cr release*		*% of total*
B	M. Brown	30:1	2	?	5:1(AS)	61	0.90	97
19	Thiele	25:1	9	?	25:1	39	1.00	77
24	Slaback	32:1	0	?	16:1	55	0.84	100
27	Rodmar	15:1	23	?	3:1	68	0.91	90
28	D. Brown	20:1	5	?	20:1	30	?	67
29	Drew	40:1	35	0.89	10:1	69	0.80	87
37	Gunnels	50:1	50	0.64	100:1	50	0.82	−44
37	Keld	100:1	7	?	25:1	35	0.71	94
				Mean: 0.77(2)			Mean: 0.85(7)	

Amount generated cytotoxicity was estimated as described in the legend to Table VII. The relative specificity index was calculated as described in Materials and Methods.

cell recognition. We have subsequently obtained additional evidence which clearly shows that Raji target cells or other SK resistant targer cells are not per se resistant to lysis. We have thus found that a 6-day pokeweed mitogen stimulation leads to the induction of killer cells which are equally, if not more, cytotoxic to Raji cells and other SK resistant target cells as compared to highly SK susceptible target cells such as Molt-4.[2] The implication of this finding is at present unclear. K cells and antibodies seem not to be involved either in the SK system or in the pokeweed mitogen generation system.

The major effort in the present paper has been to link the SK system to the B-cell line stimulation system in which killer cells are generated by cocultivation with autologous or allogeneic cells (9–13). It seems clear from the present work that B-cell line generated killer cells have a very similar target cell specificity as compared to that of the SK cells. We tentatively interpret this to mean that these two systems may be interconnected in the sense that antigens, similar to those expressed on Epstein-Barr virus transformed B-cell lines, may be involved in the in vivo generation of SK cells. Apart from the induction of cytotoxic effector cells with SK specificity, it is also clear that specific killer cells directed against the alloantigens of the stimulating B-cell line develop during the 5-day culture period (12).[3] In our present work we have not tested for cytotoxicity directed against alloantigens as we have been primarily interested in detecting killer cells with SK specificity. It is interesting though to note that Seeley and Golub when testing killer cells generated in conventional MLC cultures, find that effector cells cytotoxic to SK susceptible target cells appear before the specific killer cells and that they also disappear before the specific cells (27).

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[2] S. Targan and M. Jondal. 1978. Manuscript in preparation.

[3] C. S. Spina and J. L. Fahey. 1977. Specific and shared antigens on human lymphoid cell lines detected by induced T cell cytotoxicity. Manuscript submitted for publication.

The molecular basis underlying SK and B-cell line generated cytotoxicity is unclear and does not allow a comparison between the two systems. It is clear, however, that B-cell line generated killer cells do not express Fc receptors even when generated from Fc receptor positive lymphocytes. Already after short-term stimulation (48 h), no effector cells bind to immune complex monolayers and no ADCC effector cells can be dectected against antibody covered nucleated target cells.[4] It does thus appear as if an ADCC-like effector mechanism as suggested for the human SK system by Akire and Takasugi and Koide and Takasugi (28, 29) is less likely for the generated killer cells. In fact, we have to date no evidence in the SK system either that SK effector cells act by cytophilically absorbed IgG. Most of our present data does not support this hypothesis. For example, we find no inhibition of SK cytotoxicity with anti-Ig antibedies and no inhibition of SK cytotoxicity regeneration after trypsin treatment of lymphocytes by protein A, a molecule known to compete with cellular Fc receptors for IgG molecules.

From the present work, and earlier reports, it is clear that stimulatory antigens are expressed preferentially on B-cell lines and less, or not at all, on T-cell lines (26). This circumstance raises the possibility that HLA-D antigens may be involved, especially as it has recently been shown that autologous fresh B cells can be stimulatory. Opeltz et al., Kuntz et al., and Billings et al. have shown that the conventional MLC reaction can be inhibited with rabbit antisera directed against all known HLA-D allospecificities (30–32, R. Billings, personal communication). We have used the same antisera (kindly provided by Dr. Ron Billings, University of California at Los Angeles) and the same approach as Billings et al. to investigate this possibility but so far these antisera have not reproducibly inhibited the proliferative response in our system which possibly may be related to a higher antigen expression on the cell lines as compared to the fresh B cells.

Even if we at the present time have no evidence that B cell associated alloantigens are responsible for the proliferative response it is interesting to note that there is a similarity between the present system and the MLC reaction in the sense that the stimulation antigens and the target antigens are distinct. In the MLC reaction HLA-D serves as inducer of proliferation but not as target resembling the inverse relationship between ability to stimulate and susceptibility to cytotoxicity in the present system. Thus, B-cell lines of normal origin are good stimulators but poor targets in contrast to T-cell lines which are good targets but poor stimulators. This distinction has also quite recently been noted by Vande Stouwe et al. who found less killing of the stimulating cell line as compared to unrelated cell lines (33).

If SK cell generation is linked to B-cell associated "amplification" antigens, this would advance our knowledge about the nature of the SK system. For one thing, it is clear that these antigens are also expressed on cells belonging to the monocyte-macrophage series (34), and it is thus possible that these non-B cells are also involved in SK cell regulation. We are presently investigating this possibility. Another interesting point is that Svedmyr has shown that the proliferative response to autologous B-cell lines involves a short memory function as it is reduced during restimulation and absent during a second restimulation (12). This was in contrast to the T-cell response in conventional allogeneic MLC cultures in which responding cells became increasingly more active when repeatedly stimulated during a 4-mo period (12). If the proliferative response is involved in the generation of SK killer cells, it would thus appear that memory function in the SK system is short-term and that MLC responsive T cells and SK cells belong to different cell populations. In this context it is also interesting to note that we were able to induce killer cells from all tested subpopulations (Fig. 4 and Table VIII) even from Fc receptor negative non-SK cells. In addition to the induction of cytotoxic effector cells, we also found a strong proliferative response as measured by [^{3}H]thymidine incorporation in all tested subpopulations except in SRBC receptor negative lymphocytes (as reported in more detail elsewhere).[4] With SRBC receptor negative cells, which also are unresponsive to several mitogens (35), we furthermore found that B-cell line stimulation did increase the cytotoxic potential although the lack of proliferation correlated with the retention of Fc receptors on the effector cells.[4] The capacity of all subpopulations to respond to B-cell line stimulation by induction of SK cytotoxicity demonstrates an impressing potential of the system.

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[4] S. Targan and M. Jondal. 1978. Lack of Fc receptor expression on cytotoxic effector cells with spontaneous killer (SK) cell specificity induced by stimulation with B cell lines. Manuscript submitted for publication.

In summary, our results show that B-cell associated antigens can cause the induction of cytotoxic effector cells which have SK specificity. Furthermore, both the preformed nature of the SK system and the short memory function in the B-cell line response as compared to a conventional T-cell response may indicate a function for the SK system as a primary defense barrier in the immune response which has the capacity to act before a more conventional immune response is mounted. It is easy to conceive that there must be a great advantage for the host to have a lymphocyte cell population with a preformed "spontaneous" specificity which can deal with a low number of target cells, whether virus infected or transformed in nature, before these multiply to such a level that they can induce a B- and T-cell response. Finally, if the SK system is at all important as an anti-tumor surveillance system, one may consider the possibility to activate SK cells in vivo. If our results are taken to demonstrate in vitro SK activation, these may eventually be extended to allow a similar activation in vivo.

Summary

The present work shows that stimulation of peripheral blood lymphocytes with autologous or allogeneic B-cell lines leads to a strong induction of cytotoxic effector cells with spontaneous killer (SK) cell specificity, apart from the specific response directed against the particular stimulating cell. To demonstrate this we have determined a relative target cell specificity in the SK system, defined by the short-term ^{51}Cr release assay, and established a relative specificity index (RSI). Using this approach we have been able to show that killer cells induced during a 5-day cocultivation period with B-cell lines have a similar PSI to that of unstimulated SK cells. In addition, we have shown that such killer cells can be induced from several different lymphocyte subpopulations and that they, in

contrast to SK cells, do not express Fc receptors. The implications of these findings in relation to the nature, mechanism, and biological significance of the SK cell system is discussed.

The excellent technical assistance of Ms. Deborah Brown is gratefully acknowledged. We are also grateful to Dr. Celsa Spina for a continuous supply of lymphoid cell lines.

Received for publication 19 December 1977.

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ISOLATION OF A HETEROGENEOUS POPULATION OF TEMPERATURE-SENSITIVE MUTANTS OF MEASLES VIRUS FROM PERSISTENTLY INFECTED HUMAN LYMPHOBLASTOID CELL LINES*

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A number of slow neurological diseases occurring in animals and in man appear to be associated with a long-term, chronic infection of the host by a virus or viral-like agent. Measles virus, or a variant of measles, has been shown to be the etiologic agent of the chronic and invariably fatal human disease, subacute sclerosing panencephalitis (SSPE).[1] In SSPE, the virus appears to produce a persistent infection of both the central nervous system and lymphoid cells (1-3). There is also increasing evidence that measles virus might be associated with multiple sclerosis (MS), and possibly with other chronic human diseases as well (4).

Several observations suggest that lymphoid cells might serve as a reservoir for measles virus during the long incubation period in a chronic infection before the onset of clinical manifestations. One of the first isolations of the measles variant virus associated with SSPE was from lymph nodes of SSPE patients (3). Recently, detection of measles viral antigens in the mononuclear cells of the jejunal mucosa of patients with MS has been reported (5), and measles-like virus has been isolated from jejunal biopsies (6).

Persistently infected cultured cells have potential usefulness as models for the study of chronic diseases of viral etiology. Several mechanisms have been suggested for establishing or maintaining the carrier state. Factors such as interferon (7), conditional lethal mutants (8), defective interfering (DI) particles (9), and conceivably the production of DNA provirus copies (10, 11) might be involved. This report deals with the study in vitro of a persistent infection of human lymphoblastoid cell lines (LCL) by measles virus. Establishment and

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* Supported by Multiple Sclerosis grant 1006-B-2, and grants AI 09807 and AI 10702 from the National Institutes of Health.

‡ Supported by a National Science Foundation graduate fellowship. The data in this paper are from a thesis to be submitted in partial fulfillment of the Degree of Doctor of Philosophy in the Sue Golding Graduate Division of Medical Sciences, Albert Einstein College of Medicine, Yeshiva University.

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[1] *Abbreviations used in this paper:* CPE, cytopathological effects; DI, defective interfering; DME, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; LCL, lymphoblastoid cell line; MOI, multiplicity of infection; MS, multiple sclerosis; P. E., plating efficiency; PFU, plaque-forming units; PI, persistently infected; SSPE, subacute sclerosing panencephalitis; ts, temperature-sensitive; VSV, vesicular stomatitis virus.

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the characteristics of the persistently infected cells are described, and in addition, we report the isolation and preliminary characterization of a highly heterogeneous population of temperature-sensitive (ts) mutants produced by the carrier cultures.

Materials and Methods

Cells. The human LCL WI-L2 and 8866 were propagated as suspension cultures in RPMI-1640 medium (Grand Island Biological Co., Grand Island, N. Y.), supplemented with 17% fetal calf serum (FCS; Flow Laboratories, Inc., Rockville, Md.), and glutamine. Both WI-L2 and 8866 are presumably transformed by Epstein-Barr virus, and carry markers characteristic of human B lymphocytes (12).

Monolayer cultures of Vero cells, derived from green monkey kidney cells, were used for all viral isolations, titrations, and characterization assays. They were the gift of Dr. Philip Marcus, University of Connecticut, Storrs, Conn. Cultures were grown in Dulbecco's modified Eagle's medium (DME; Grand Island Biological Co.) supplemented with 10% FCS and glutamine. All cultures were incubated in a humidified atmosphere of 5% CO_2 in air, at 37°C unless otherwise noted.

Viruses. The Edmonston strain of measles virus was originally obtained from Dr. Bernard Fields. Parental virus was doubly plaque-purified on Vero cells, and stock supernatant virus was produced by growth in Vero cells at a multiplicity of infection (MOI) of 0.01 plaque-forming units (PFU)/ml. Supernatant virus was then separated from cell debris by sonication, followed by clarification of the supernate by low-speed centrifugation. The stock virus was stored in 10% dimethylsulfoxide at −60°C, and contained 5×10^6 PFU/ml by plaque assay on Vero cell monolayers at 37°C. The Indiana (HRC) strain of vesicular stomatitis virus (VSV) was the gift of Dr. Philip Marcus.

Virus Titrations. Virus preparations were titered by a semiquantitative plaque assay (13). Vero cell monolayers were trypsinized and plated in 96-well plastic trays (Linbro Scientific Co., Hamden, Conn.). Approximately 1.3×10^5 cells were plated in each 16-mm well and incubated at 37°C overnight. 10-fold dilutions of virus samples were made in DME with 2% FCS. The medium from each well was aspirated, and duplicate wells were inoculated with 0.2 ml of viral dilutions. After an adsorption period of 2 h at 31°C, 1 ml of growth medium containing 0.75% carboxymethylcellulose was added. The monolayers were then incubated at the appropriate temperature for 5 days, then fixed with 10% formyl saline and stained with crystal violet.

Persistent Infection of LCL. The persistent infection of the LCL was established using a modification of the method of Barry et al. (14). Briefly, 2.5×10^6 lymphoblastoid cells were infected at an MOI of $1\text{–}4 \times 10^{-6}$ PFU/cell. The inoculum used was a 10% hamster-brain suspension, derived from neonatal hamsters injected intracerebrally with parental virus and containing 10^6 PFU/g of tissue. Medium from infected cultures grown at 37°C was changed weekly, and the cultures were able to be passaged ≅ 5 wk after infection.

Immunofluorescence Staining. Measles viral membrane antigens were detected by the indirect staining technique. Cells in suspension were stained with a rabbit anti-measles serum produced in this laboratory, followed by staining with rhodamine-conjugated goat anti-rabbit serum (N. L. Cappel Laboratories Inc., Cochranville, Pa.). The cells were washed, dried on glass slides, post-fixed in absolute ethanol, mounted in buffered glycerol, and examined microscopically with ultraviolet illumination. Measles viral cytoplasmic antigens were detected by direct staining of cells dried onto glass slides and prefixed with absolute ethanol. The fixed cells were then stained with fluorescein-conjugated rabbit anti-measles serum diluted in phosphate-buffered saline containing 80% FCS and 0.5% Evans blue counterstain. Cells were washed extensively, mounted in buffered glycerol, and examined by ultraviolet illumination.

Electron Microscopy. Cells were pelleted and fixed in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3. The cells were then post-fixed in 1% OsO_4, dehydrated in a graded series of ethanol, and embedded in Araldite-Epon. Sections were cut, stained with lead citrate-uranyl acetate, and examined with a Siemons 102 electron microscope (Siemons Corp., Cherry Hill, N. J.).

Superinfection of Persistently Infected (PI) LCL. WI-L2 and WI-L2$_{PI}$ I were tested for resistance to infection by VSV(HRC) or by parental measles virus. Each cell line was either infected with VSV at an MOI of 1 or 10, or with measles virus at an MOI of 0.01. VSV-infected and uninfected cultures were incubated at 37°C for 40 h, and samples were removed for viable cell count by trypan blue exclusion, and for assessing the amount of VSV produced by titration on Vero cells. The amount of DNA synthesis was also measured in surviving cells by uptake of $[^3H]$thymidine (New England Nuclear, Boston, Mass.) in a microtiter assay for 18 h. Quadruplicate samples were labeled and collected on a multiple cell culture harvester. Measles-infected and uninfected control cultures were incubated at 37°C for 6 days before samples were removed for viable cell counts and assessing yields of measles virus.

Transfection Studies with DNA from PI LCL. DNA was isolated from $\cong 10^8$ WI-L2$_{PI}$ I cells by the method of Marmur (15). Transfection of Vero cell monolayers was performed by the calcium precipitation technique of Graham and Van der Eb (16). Treated cells were incubated at 33°C for 14 days and examined for evidence of cytopathology.

Interferon Assay. Supernates of PI and control cultures were assayed for interferon production by the interference assay of VSV replication on human foreskin cultures (17). Any measles virus present was removed by ultracentrifugation or by adjusting the supernates to pH 2–3.

Production and Quantitation of DI Particles. Measles DI particles were produced and amplified by multiple undiluted passages of parental virus in Vero cell monolayers. Samples were assayed for interference by mixing 0.1 ml of serial twofold dilutions of a DI preparation with 0.1 ml of a preparation containing parental measles virus. The mixtures were inoculated in duplicate onto Vero cell monolayers in plastic trays (Linbro Scientific Co.) containing 24 wells of 16-mm diameter. Control wells received 0.1 ml of each twofold dilution of the DI preparation, or 0.1 ml of the parental virus. Samples were incubated and scored as in the virus titration assays.

Plaque Purification of Viral Isolates from PI LCL and Screening for ts Mutants. PI cell cultures were grown at 31°C for 24–48 h. The supernates from these cultures were harvested, filtered through a 0.45-μm Millipore filter to remove viral aggregates, and plated on Vero monolayers in 60-mm Petri dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). An appropriate dilution of the filtered supernates was used to give 25–50 plaques per dish. After a 2-h adsorption period at 31°C, the monolayers were overlaid with 4 ml of modified Eagle's medium containing 10% FCS and 0.75% agarose. Plates were incubated at 31°C for 7–10 days, then overlaid again with 2 ml of agarose medium containing 0.00125% neutral red, and incubated for an additional 18 h at 31°C. Well-isolated plaques were selected and inoculated onto individual Vero monolayers in 16-mm Linbro tray wells, with the addition of 1 ml of growth medium. The plaque-purified isolates were incubated at 31°C and harvested when 90% of the monolayer showed cytopathology (CPE). The entire culture fluid (with cell debris) was then inoculated onto Vero monolayers in 25 cm^2 Falcon flasks (Falcon Plastics), incubated at 31°C, and harvested when 90% of the monolayer showed CPE. Each isolate was then screened for temperature sensitivity by plaque assay at 31 and 39°C. A plating efficiency (P.E.) ratio for each isolate was calculated as follows:

P.E. ratio = 39°C titer/31°C titer.

Thermolability of ts Mutant Isolates. Thermolability was determined by measuring the rate of reduction in PFU/ml at 31°C after incubation of virus samples at 45°C. Undiluted virus stocks were incubated in 12-ml glass conical centrifuge tubes in a 45°C water bath. Samples (0.2 ml) were withdrawn into 1.8 ml of ice-cold DME containing 2% FCS at 0, 15, 30, and 45 min. Residual infectivity was determined by plaque assay at 31°C.

Results

Establishment of PI Cell Cultures. Persistent infection of LCL was initiated by a modification of the method of Barry et al. (14) by infection of cultures at an MOI of 10^{-6}. Even at this low input MOI, a crisis ensued, and the majority of the cells in the culture lysed as in an acute infection. The surviving cells were passaged $\cong$5 wk after infection. The three PI LCL to be described here were independently established: WI-L2$_{PI}$ I, WI-L2$_{PI}$ II, and 8866$_{PI}$. In the 2 yr since establishment of the PI cultures, the LCL have remained stable. No cycles of crisis and recovery have been observed.

TABLE I

Characteristics of Measles PI Lymphoblastoid Cell Lines

Characteristic	Cell lines		
	WI-L2$_{PI}$ I	WI-L2$_{PI}$ II	8866$_{PI}$
Immunofluorescent staining*	70–80%	70–80%	90%
Electron microscopy	many cells with virus budding, nucleocapsid matrices in cytoplasm	ND‡	ND
Infectious centers§	1–2%	2–3%	1–2%
Supernate viral yield (PFU/ml)‖	1	1–2	1–2
Intracellular viral yield (PFU/ml)	1–2	1–2	1–2
Interferon (U/ml)¶	291	57	49

* Percent of cells in culture containing membrane viral antigen by indirect immunofluorescent staining.

‡ ND, not done.

§ Percent of cells in culture producing infectious virus.

‖ PFU/infectious center.

¶ Uninfected control WI-L2 had less than 10 U/ml. Uninfected control 8866 had 88 U/ml.

Properties of PI LCL. The PI lines appear morphologically normal, with fewer than 1% giant cells in the cultures. The LCL were examined soon after establishment for the presence of measles virus. The results (Table I) confirm previous reports from other laboratories (14, 18) that the cultures were PI. Immunofluorescent staining (Fig. 1) and electron microscopy (Fig. 2) indicated that the vast majority of the cells in each infected culture were producing measles viral antigens and virus-like particles. However, an infectious centers assay (19) showed that only 1–2% of the cells were producing infectious virus at 37°C. In addition, very few infectious viral particles could be detected by plaque assay on Vero cells. Only 1–2 PFU were released into the supernate from each infectious center ($\cong 10^4$ PFU/ml). When cells were disrupted by freezing and thawing, each productive cell again released only 1–2 infectious particles.

By immunofluorescence, the PI LCL cultures appeared heterogeneous in the intensity of staining for measles cytoplasmic antigens (Fig. 1). One line, WI-L2$_{PI}$ I, was cloned by the soft agarose technique (20). 23 cell clones were grown up and screened for membrane antigen by the indirect or direct staining method. 2 of the 23 clones were negative for viral antigens. The remaining 21 clones exhibited a homogeneous staining pattern in the cells within each clone. But a range of intensity of staining among the clones was seen, from faintly positive clones to clones that were highly fluorescent. One intensely fluorescent cell line, cell clone 16, was chosen to be doubly cloned for further study.

Possible Factors Involved in Maintenance of the Carrier State

INTERFERON. Some LCL have been shown to secrete continuously low levels of interferon (21), although they appear to be relatively resistant to the antiviral effects of interferon (22). A rise in interferon levels was seen in the supernates of both PI lines derived from WI-L2 (Table I), from a base level of <10 U/ml in the control culture. In contrast, the level of interferon declined

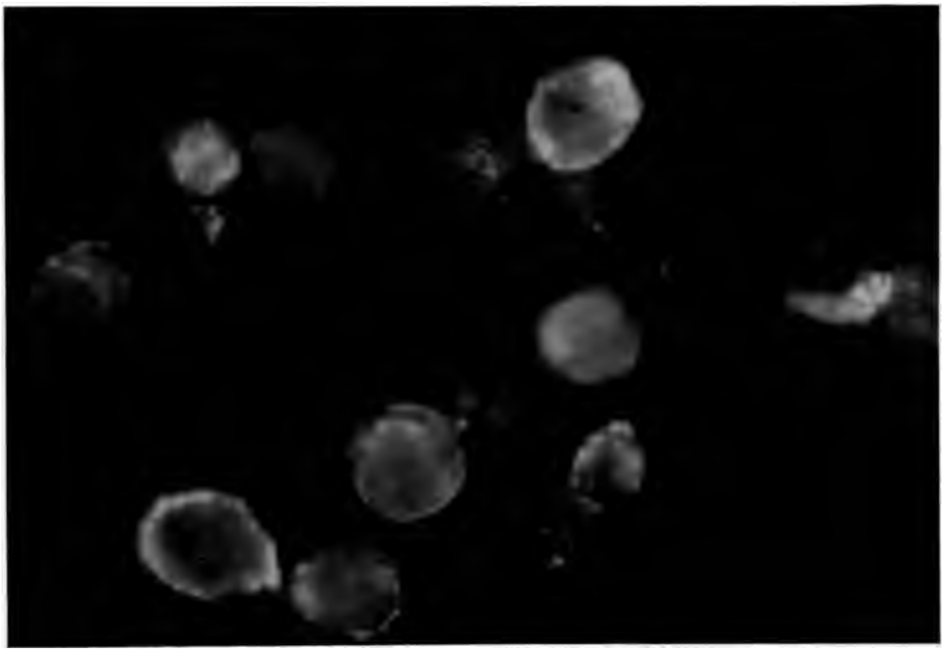

FIG. 1. Measles viral antigens in the cytoplasm of WI-L2$_{PI}$ I cells, by direct immunofluorescent staining of alcohol-fixed cells with fluorescein-conjugated rabbit anti-measles serum in Evans blue counterstain. Magnification, 100 ×.

when 8866 became PI (from 88 to 49 U/ml). In these experiments, although an increased production of interferon was not always associated with a persistent measles infection in the LCL, we cannot exclude the possibility that the low levels of interferon produced by uninfected cells were sufficient to establish the carrier state.

If interferon were a major factor in maintaining the carrier state, one might expect the PI LCL to be resistant to infection by other viruses. The resistance of WI-L2$_{PI}$ I to challenge with VSV(HRC) or measles virus was determined (Tables II and III). Both control WI-L2 and WI-L2$_{PI}$ I were susceptible to infection by VSV, as indicated by the viral yields and by CPE as assayed by trypan blue exclusion. Cell clone 16 of WI-L2$_{PI}$ I was also as susceptible as control WI-L2 cells to VSV, as measured by reduced uptake of [^{3}H]thymidine in infected cultures (Table III).

When challenged with the parental measles virus, the PI LCL exhibited homologous interference (Table II). Yields of measles virus from unchallenged and superinfected WI-L2$_{PI}$ I were comparable, and were lower than the yields obtained from control WI-L2 cells infected with measles. The control cells also showed extensive cell destruction (>90% nonviable by trypan blue exclusion), whereas a much lower amount of CPE was seen with the superinfected WI-L2$_{PI}$ I culture (30% nonviable cells).

DNA PROVIRUS COPIES. It has been suggested by Simpson and Ilnuma (10) and Zhdanov (11) that enveloped RNA viruses could maintain a persistent viral infection by the production and integration into host cell DNA of a DNA

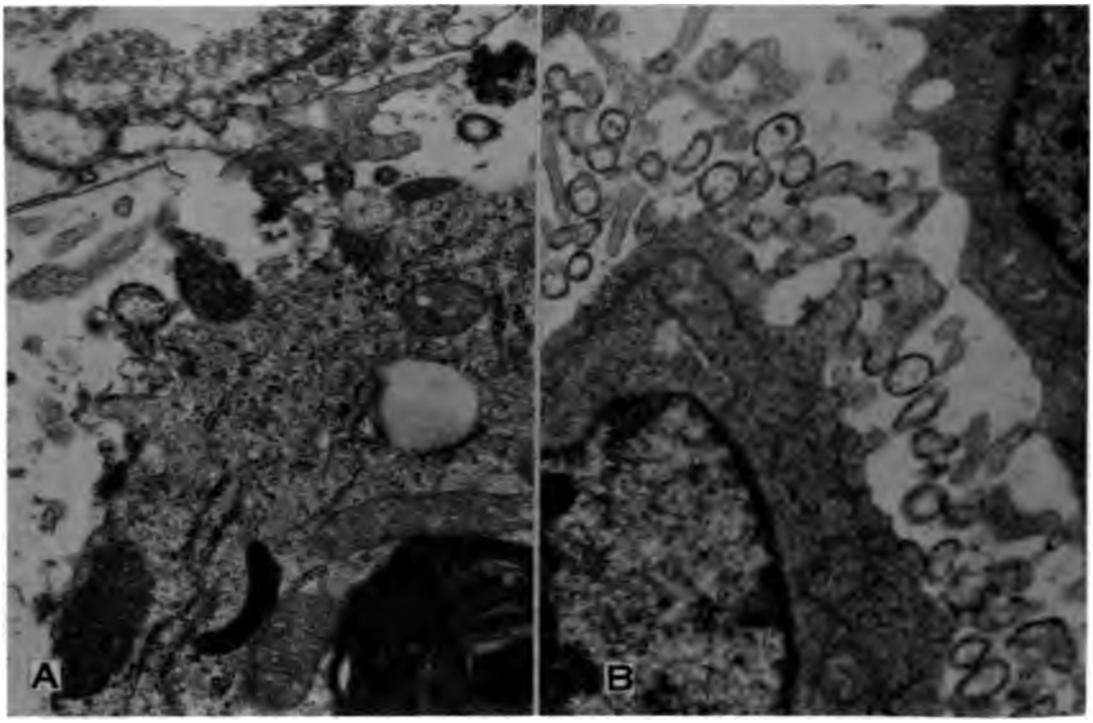

FIG. 2. Electron photomicrographs of lymphoblastoid cells PI with measles virus. (A) WI-$L2_{PI}$ I cells with aggregates of viral nucleocapsids aligned along the cell membrane. Magnification, 24,000 ×. (B) Measles virus-like particles released from WI-$L2_{PI}$ I cells. Magnification, 30,000 ×.

TABLE II
Superinfection of WI-L2 and WI-$L2_{PI}$ I with VSV and Measles Virus

Cell line	Virus*	Time after infection	Viable cell count	CPE‡	Supernatant virus yields§	
					PFU/ml	PFU/10^6 viable cells
			cells/ml	%		
WI-L2	VSV	40 h	4.7×10^5	50	2.5×10^7	5×10^7
WI-$L2_{PI}$ I	VSV	40 h	6×10^4	80	1×10^7	1.6×10^6
WI-L2	measles	6 days	1.6×10^5	>90	6.2×10^3	4×10^4
WI-$L2_{PI}$ I	measles	6 days	4.8×10^5	30	2×10^3	6.5×10^3
WI-$L2_{PI}$ I	none	6 days	8×10^5	<1	5×10^3	4.2×10^3

* VSV: MOI = 1; measles: MOI = 0.01.

‡ Percent of cells that did not exclude trypan blue.

§ Assayed by plaque titration on Vero cells at 37°C.

provirus. Transfection studies using DNA extracted from WI-$L2_{PI}$ I cells were negative for production of measles viral CPE on Vero cells at 33°C. Similar negative results have been reported by Holland et al. (23) for cells persistently infected with VSV, measles, rabies, or mumps (23).

DI PARTICLES. Since persistent infection by several other RNA viruses has

TABLE III
Superinfection with VSV

Cell line	MOI	[^{3}H]Thymidine incorporation*		Viable cell count
		cpm	Uninfected	
			%	*cells/ml*
WI-L2	0	265,469	100.00	1.9 × 10^6
	1	338	0.13	<10^4
	10	309	0.12	<10^4
WI-L2$_{PI}$ I, clone 16	0	159,543	100.00	1 × 10^6
	1	373	0.23	<10^4
	10	333	0.21	<10^4

* 0.2 ml of cell cultures was incubated with 1 μCi of [^{3}H]thymidine per well in microtiter plates for 18 h at 37°C, on day 3 post-infection. Each value is an average of four wells.

been shown to be mediated by DI particles (9, 23, 24), the PI LCL were examined for the production of particles with interfering activity. A stock DI-enriched preparation of measles was produced by seven undiluted passages of parental virus. This stock contained no PFU when assayed on Vero cells at 37°C, and 0.1 ml of this sample reduced the yield of parental measles virus by at least one log of infectivity. Attempts were made to demonstrate the presence of DI particles in cell supernates. No evidence was obtained for the presence of significant numbers of DI particles in fluids harvested from PI LCL. Undiluted PI supernates harvested from WI-L2$_{PI}$ I or from 8866$_{PI}$ (grown at 31°C or at 37°C) failed to produce any interference in the replication of the stock measles virus at 37°C. The interference assay was also performed with undiluted supernates passaged one, two, or three times on Vero cell monolayers at 31°C. These cycles of amplification for DI particles did not result in any detectable interference with the parental measles replication.

TS VIRAL MUTANTS. Persistent infection of a variety of mammalian cell lines has been reported to be associated with the appearance of ts mutants (8). Recent studies have demonstrated a similar finding in several cell lines persistently infected with measles (25–28). Therefore, the LCL cultures were examined for the appearance of ts mutants. The P.E. ratio of total progeny virus from the PI cells did not differ significantly when titrated at 31, 37, or 39°C (data not shown). The possibility remained that ts mutants might exist as a fraction of the total viral population. This was evaluated by plaque purification of virus from filtered supernates of PI cultures grown at 31°C for 24–48 h. Individual isolates were propagated on Vero cells, and titered by the plaque assay at 31 and 39°C. The P.E. ratios (39°C titer/31°C titer) were determined for viral plaque-purified isolates from the three parental cell lines (Table IV). Each of the parental cell lines, WI-L2$_{PI}$ I, WI-L2$_{PI}$ II, and 8866$_{PI}$, produced a population of viral isolates with a range of P.E. ratios from $>10^{-1}$ to $<10^{-4}$.

The appearance of a heterogeneous population of ts mutants could have been attributed to the heterogeneity of the cell population, with different cells producing a particular ts mutant. Therefore, cell clone 16, a doubly cloned cell line of WI-L2$_{PI}$ I, was also examined for the production of ts mutants by plaque purification. Again, isolates with a range of P.E. ratios were obtained, suggest-

TABLE IV

Ratios of P.E. at 39 and 31°C of Viral Isolates from Supernates of PI LCL and of Parental Stock Virus

	Cell Line					
	WI-L2$_{PI}$ I	WI-L2$_{PI}$ II	8866$_{PI}$	Clone 16	Total	Parental stock
Total number of isolates	17	15	17	21	70	21
Number of isolates with P.E. ratios*						
$1-10^{-1}$	3(18%)‡	8(53%)	7(41%)	1(5%)	19(27%)	21(100%)
$10^{-1}-10^{-2}$	2(12%)	4(27%)	7(41%)	5(24%)	18(26%)	0
$10^{-2}-10^{-3}$	2(12%)	2(13%)	0	6(29%)	10(14%)	0
$10^{-3}-10^{-4}$	7(41%)	1(7%)	0	7(33%)	15(21%)	0
$10^{-4}-10^{-5}$	3(18%)	0	3(18%)	2(9%)	8(11%)	0

* P.E. = ratio = 39°C titer/31°C titer.

‡ (%) = number of isolates with a given P.E. ratio/total number of isolates × 100.

ing that even a clone of lymphoblastoid cells could produce a heterogeneous virus population. In Table IV are presented the P.E. ratios of 70 viral isolates from the 4 PI LCL. P.E. ratios greater than 10^{-1} were found with 19 of the 70 isolates, but the majority of the plaque-purified isolates (51/70) showed at least some degree of temperature sensitivity. It was also observed during the plaque purification procedure that isolates differed in plaque size and morphology. However, this phenotypic marker did not breed true, and plaque size did not correlate with P.E. ratios (i.e., virus from an initially small plaque did not always exhibit a low P.E. ratio).

To discount the possibility that viral mutants were present in the original inoculum, parental measles virus was grown at 31°C and plaque-purified by the same method as used with the PI LCL supernates. When 21 viral isolates were examined, all 21 had a P.E. ratio of 1 (Table IV). This suggests that the appearance of ts mutants in the stock virus is not a high frequency event, and that the population of ts mutant virus was induced during the establishment of the persistent infection, not selected from preexisting mutants.

To examine the question of whether or not the plaque-purified isolates were stable viral mutants, isolates from cell clone 16 were tested by a second plaque purification (Table V). Two viral isolates with initial P.E. ratios of $<10^{-4}$ and two isolates which appeared to be leaky mutants (P.E. ratios $>10^{-1}$) were selected. All plaque-purified isolates of 4M and 9S retained the ts phenotype, exhibiting P.E. ratios comparable to those of the original viral stocks. However, the viral isolates 1S and 7M generated plaques with a range of P.E. ratios from 10^{-1} to 10^{-5}. These two initially leaky isolates were either highly unstable, or consisted of a mixed population of virions. Isolates from 1S are being examined after a third plaque purification for the ability to retain their initial P.E. ratios.

Thermolability of the ts Mutants. A ts mutation affecting a structural component of the virion might in some cases result in altered thermolability of the mutant virus. Therefore, the kinetics of heat inactivation of several of the ts isolates were compared to those of the parental virus. The parental measles virus was completely inactivated after 60 min at 45°C (Fig. 3). Seven viral

TABLE V

Ratios of Plating Efficiencies of Viral Isolates after a Second Plaque Purification

	Viral isolate			
	1S	7M	4M	9S
Initial P.E. ratio	10^{-1}	10^{-1}	10^{-4}	10^{-4}
Total no. of isolates	7	11	11	12
Number of isolates with P.E. ratio				
10^{-1}-10^{-2}	1(14%)	0	0	0
10^{-2}-10^{-3}	1(14%)	1(9%)	6(55%)	0
10^{-3}-10^{-4}	3(43%)	9(82%)	5(45%)	11(92%)
10^{-4}-10^{-5}	2(29%)	1(9%)	0	1(8%)

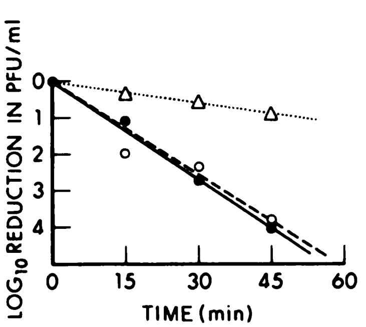

FIG. 3. Thermolability of parental and ts isolates of measles virus at 45°C. Undiluted virus stocks were incubated in 12-ml glass conical centrifuge tubes in a 45°C water bath. Samples (0.2 ml) were withdrawn into 1.8 ml of ice-cold medium containing 2% FCS at 0, 15, 30, and 45 min. Residual infectivity was determined by plaque assay at 31°C. Parental virus (●), ts isolate 12 (○), and ts isolate 9S (△).

isolates from two of the PI LCL (representing a range of P.E. ratios from 10^{-1} to 10^{-5}) were tested and could be divided into two groups. One group, which included five of the seven isolates tested, showed a rate of inactivation similar to that of the parental virus. The thermolability curve of isolate 12 (from cell line 8866_{PI}) is representative of this first group. Two other viral isolates appeared to be more thermostabile than the parental virus, as has been reported with virus from another measles PI cell line (26). The inactivation curve of one of these isolates, 9S from cell clone 16, is also shown. The thermolability of the isolates did not correlate with the P.E. ratios, since both isolates 12 and 9S had P.E. ratios $<10^{-4}$. The data on kinetics of heat inactivation suggest that there exist at least two groups of ts mutants that can be distinguished by comparison of their thermolability at 45°C, again confirming the heterogeneity of mutants produced by the PI lines.

Measles Antigen Production by ts Mutants at 39°C. The amount of measles-specific antigens synthesized by several ts isolates at the nonpermissive temperature was assayed by direct immunofluorescence on alcohol-fixed infected cells. When parental virus was used to infect Vero cells at 39°C for 12 h, extensive cell destruction could be seen in the monolayer. Immunofluorescent staining showed intense fluorescence in the cytoplasm, with some antigen

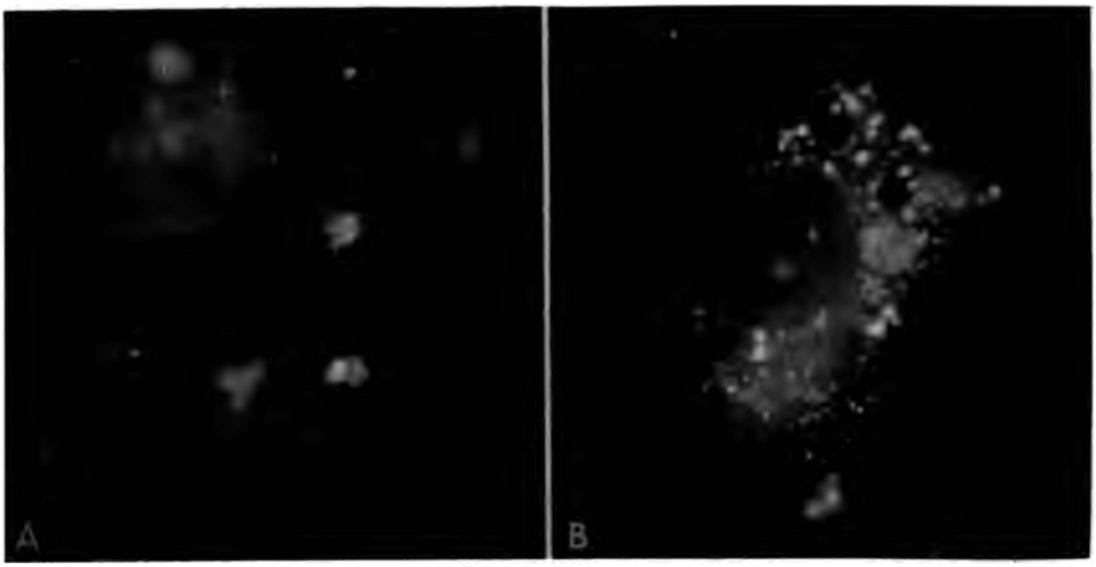

FIG. 4. Differences in antigen production at the nonpermissive temperature (39°C) by ts isolates of measles virus. Vero cell monolayers were infected at an MOI of 1-2 with individual mutants, and incubated at 39°C for 3 days. Monolayers were washed, alcohol-fixed, and stained with fluorescein-conjugated rabbit anti-measles serum in Evans blue counterstain. (A) Vero cells infected with isolate 8M from WI-L2$_{PI}$ II, showing faint cytoplasmic fluorescence. Magnification, 100 ×. (B) A single Vero cell infected with isolate 15M from 8866$_{PI}$, showing intense cytoplasmic fluorescence. Magnification, 100 ×.

localized in the nucleus. Control uninfected Vero cells exhibited no specific measles fluorescence. Four ts isolates, one each from WI-L2$_{PI}$ I, WI-L2$_{PI}$ II, clone 16, and 8866$_{PI}$, were selected for study. All four isolates had P.E. ratios $<10^{-5}$. Infection of Vero cells was initiated at an MOI of 1-2 by a 2-h adsorption period at 31°C. Growth medium was then added and the cultures were incubated for an additional 4 h at 31°C to allow penetration. The infected monolayers were then shifted up to 39°C and incubated for an additional 3 days.

None of the monolayers infected with ts mutants showed any visible signs of CPE. With three of the four mutants, the pattern of antigen production indicated that these isolates were early mutants, synthesizing only a very small amount of measles proteins (Fig. 4A). Monolayers infected with the fourth mutant (15 M from 8866$_{PI}$) showed a staining pattern resembling that of the parental virus, with intense cytoplasmic fluorescence and a faint nuclear staining (Fig. 4B).

The range in P.E. ratios of the ts isolates has been accompanied by differences in thermolability and in antigen production at the nonpermissive temperature. It is probable, therefore, that the mutants that exhibit phenotypic differences also represent a genotypically mixed population. By genetic complementation analysis, we are examining whether or not the ts isolates from different cell lines have lesions in different virus genes.

Attempts to Cure the PI LCL. The inclusion of rabbit anti-measles serum to the culture medium was examined for its effect on the persistent infection of WI-L2$_{PI}$ I. Antibedy (0.5 ml of an undiluted serum), capable of neutralizing 10^{8}

PFU, was added to 10 ml of medium at each cell passage. After 3 mo of culture in anti-measles serum, the cells were grown for 1 wk without antibody and then assayed by indirect immunofluorescence staining for measles membrane antigens. No difference in fluorescence pattern was observed from the untreated PI cells; >80% of the cells remained positive for viral membrane antigens. The failure to cure the PI LCL with antibody to measles implies that the role of extracellular virus is not an important one in maintaining the carrier state.

To study the effects of temperature shifts on antigen expression, cultures of WI-L2$_{PI}$ I were shifted from 37°C, the usual incubation temperature, to 39°C, and grown at this temperature for 1 mo. No CPE was seen in the shifted cultures, and indirect immunofluorescence revealed that the vast majority of the cells still expressed measles membrane antigens. When cultures of the PI LCL were incubated at 31°C, the cells showed extensive CPE and were usually destroyed. Cultures that did survive the crisis at 31°C continued to express measles membrane antigens. Preliminary experiments suggest that some ts mutant viruses can interfere with replication of both wild-type and other mutant viruses.

Discussion

PI cultured cells have potential usefulness as models for the study of chronic diseases of viral etiology (29). Measles virus has been associated with at least two slow neurological diseases in man characterized by demyelination, SSPE and MS (1–4). Interest in measles virus latent infection has resulted in the generation of a variety of mammalian cell lines persistently infected with measles, including HeLa (27, 30, 31), HEp-2 (26), the human lung line Lu 106 (32), monkey kidney cell lines Vero (33) and CV$_1$ (34), and human lymphoblastoid cell lines (14, 18, 28).

The study of PI LCL is of particular interest because of the characteristics of lymphocytes with respect to virus infection. First, resting lymphocytes are restrictive for virus growth and readily harbor virus in a latent state (35). But lymphocytes activated by mitogens or specific antigens are then able to support the replication of several viruses, including VSV (36), measles (37), mumps (38), vaccinia (39), and Herpes simplex virus (40). Second, infection by a number of viruses including measles and mumps causes transient immunosuppression in the host (41). This temporary period of immunologic incompetence could provide the virus with an escape from the host's immune defenses and allow a persistent infection to be established in lymphoid or other cells. Third, measles virus has been isolated from lymphocytes obtained from patients months and sometimes years after an acute measles infection. Recovery of measles virus has been obtained by cocultivation on susceptible cell monolayers without mitogenic stimulation (42). Lymph node biopsies provided some of the first isolates of the measles variant implicated in SSPE (3). In addition, localization of measles antigen in mononuclear cells of jejunal biopsies of MS patients has been reported recently (6). Since some lymphocytes are known to be extremely long-lived (43), these observations suggest that lymphoid cells might serve as a reservoir for harboring the viral agents in chronic infections before the appearance of clinical symptoms and disease (35).

The PI LCL differ from other measles infected cell lines in several important characteristics. The carrier state in LCL is more readily and frequently established than in other cells, with the LCL cell culture stabilized 5 wk after infection. In contrast, a PI CV_1 line was first passaged 260 days after infection (34). Antibody to measles in the culture medium is not required, as in the case of the HEp-2 (26) measles PI cultures, nor was the addition of DI particles necessary (33). In some of the measles PI lines, no infectious virus can be detected (34). When infectious virus has been previously isolated from other cell lines, some degree of temperature sensitivity has been noted (25–27, 32), but the progeny virus were assumed to be homogeneous, and no clonal analysis was attempted. Previous reports of the PI LCL did not mention any ts characteristics of the virus being produced by the PI cultures (14, 18), and although Minagawa et al. (28) reported a slight temperature sensitivity (P.E. ratio $>10^{-2}$) when total supernatant virus was titered, this P.E. difference was not considered significant.

It is important to note that a plating efficiency ratio for total supernatant virus may be misleading in determining the presence or absence of ts mutants in a given virus population. Heterogeneous populations of virions with different P.E. ratios can become aggregated and possibly self-complementary, masking the appearance of temperature sensitivity. Clonal analysis is necessary to reveal the fraction of ts mutants in a total viral population. We have shown that a clonal analysis of progeny virus from measles-infected LCL reveals that the majority of virus produced was ts. The population of ts mutants was strikingly heterogeneous in P.E. ratios, as well as in biological assays for thermolability and antigen production at the nonpermissive temperature.

Clonal analysis has been carried out for progeny virus from a VSV-PI L cell line (44). The 34 VSV clones from this mouse cell line also showed a range of P.E. ratios from 10^{-3} to $<4 \times 10^{-7}$. However, all 34 ts clones were defective in RNA synthesis at the nonpermissive temperature. Complementation analysis indicated that the eight mutants tested belonged to complementation group I, the most common mutant class that appears among spontaneous mutants in wild-type VSV populations. Complementation analysis of the ts isolates from the PI LCL is in progress, and analysis of RNA synthesis at 39°C indicated that both RNA-positive and RNA-deficient mutants are present in the ts viral population. Therefore, preliminary results suggest that these ts mutants will represent genetic differences, and are the result of lesions in different viral genes.

Although ts mutant viruses are the most outstanding characteristics of the LCL carrier state, other factors involved in persistent infection, such as interferon and DI particles, cannot yet be excluded. Interferon and DI particles may be more important in the period before establishment of a stable carrier culture, rather than in the maintenance of persistence, as has been suggested (44).

The origin of the heterogeneous population of ts mutants is an intriguing question. It seems unlikely that multiple ts mutants could be present in the initial inoculum, because of the low MOI used (1 PFU/10^6 cells) and the failure to detect a high frequency of ts mutants in the parental virus stock used for infection. Therefore, it is probable that mutations were generated in the multiple rounds of replication during the period of 5 wk before the cultures stabilized. The phenotypic differences in the ts isolates suggest that no single class of mutants dominated, and that infection of LCL can induce a variety of mutations. Whether or not a single cell can produce a heterogeneous population of mutants is under investigation; but the likelihood of this is suggested by the clonal analysis of progeny virus from the doubly cloned culture of cell clone 16.

It is our hope that the LCL may prove useful in the generation of ts mutants, probably of different complementation classes. Persistent infection of LCL might be a close reflection of what can occur in vivo, and the study not only of ts mutants derived by chemical mutagenesis (45, 46), but also of those which arise spontaneously by persistent infection of lymphoid cells may be of importance.

Temperature-sensitive viral mutants have been useful in the study of chronic diseases. While mice infected with wild-type reovirus Type 3 develop an acute, lethal encephalitis, animals infected with some ts mutants develop a slow neurological disease (47). Inoculation of ts mutants of measles virus intracerebrally into neonatal hamsters can result in hydrocephalic changes of the brain (48). Several mechanisms based on persistence of virus in lymphoid cells could explain the development of a slow neurological disease. There could be escape of mutant virus or of wild-type revertants from the reservoir of persistence to a susceptible neurological site. It is also possible that PI lymphocytes might migrate into the central nervous system, with initiation of an inflammatory immune response against the viral antigens or infected cells, leading to demyelination or other pathological lesions as a bystander effect (49).

Whereas the viral etiology of MS is by no means established, and its association with a measles-like virus remains even more tenuous, the study of viral mutants could provide insight into one of the most puzzling aspects of this disease, namely the unpredictable exacerbations and remissions. Based on an analogy with perhaps the most classical relapsing infectious disease, African trypanosomiasis (50), one mechanism explaining exacerbations would be the emergence of variants with altered antigens. If a slow neurological disease like MS were produced by a measles-like agent, it is possible that the initial persistence of virus would involve the generation and establishment of mutant viruses. We would suggest that subsequent exacerbations might be precipitated by the emergence of antigenically altered secondary or tertiary mutants which could flourish for short periods until an appropriate primary immune response developed to suppress them and induce remission. The feasibilty of such a model for a slow neurological disease has been elegantly demonstrated recently for chronic demyelination produced in sheep by successive, antigenically variant mutants of visna virus presumably selected for by the immune response to the initial antigenic type (51).

Summary

Two human lymphoblastoid B-cell lines, WI-L2 and 8866, were infected with the Edmonston strain of measles virus at a multiplicity of infection of 10^{-6}, and stable persistent infections were established. By immunofluorescence and electron microscopy, the vast majority of cells from both cell lines were expressing viral antigens and releasing virion-like particles. However, very little infectious virus could be detected at 37°C, either by an infectious centers

assay or by titration of supernates from persistently infected cultures. When cultures were shifted to 31°C, the cells released a population of virus that was temperature-sensitive. Clonal analysis of supernatant virus at 31°C revealed a highly heterogeneous population of temperature-sensitive mutants, differing in plating efficiency ratios, thermolability, and antigen production at the nonpermissive temperature. Factors such as interferon, defective interfering particles, and extracellular virus do not appear to be important in maintaining the persistent carrier state. These studies have important implications for persistent infections of lymphoid cells in vivo, and the slow neurological diseases associated with measles, subacute sclerosing panencephalitis, and multiple sclerosis.

We thank Doctors B. Fields and R. Cross for their helpful discussion, and Ms. Yvonne Kress for her electron microscopy and photographic assistance.

Received for publication 13 February 1978.

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THREE GENES FOR LUPUS NEPHRITIS IN NZB × NZW MICE

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NZB × NZW hybrid mice provide an excellent model of spontaneous autoimmune disease, showing a very high incidence of a renal disorder which closely resembles lupus nephritis (1). The occurrence of an autoimmune condition, causing early death from renal failure, in the hybrid of two in-bred strains which do not themselves show the disorder provides a unique opportunity for elucidating the genetic basis of inherited autoimmune disease. The NZB × NZW renal disease must depend on the action of at least two genes, one from each strain, and these genes must be dominant or codominant in that they express their effect in the heterozygous state.

In an earlier paper (2) we reported the results of a study of the genetic contribution of the NZB mouse to the renal disease of the NZB × NZW hybrid, demonstrating that the NZB strain contributes a single gene or cluster of closely-linked genes to the disorder. In this paper we report a study of the genetic contribution of the NZW strain to the renal disease of the hybrid.

Materials and Methods

Mice. All mice were derived from the Otago University inbred colonies. Virgin female mice were used in all the studies described. Female (NZB × NZW)F_1 hybrid mice were backcrossed to NZB mice and 150 offspring were *H-2* typed and monitored for the onset of renal disease by using the criteria described below. In addition (NZB × NZW)F_1 mice were out-crossed to NZC mice and 190 offspring were similarly studied. Three control groups, comprising 143 (NZB × NZW)F_1 hybrids, 75 NZB, and 40 NZW mice were also monitored for the occurrence of renal disease.

H-2 Typing. The H-2 phenotype of each (NZB × NZW)F_1 × NZB mouse and of 100 of the (NZB × NZW)F_1 × NZC mice was determined by using a modification of Stimpfling's PVP hemagglutination technique (3) as described previously (2). Anti-H-2.4 and anti-H-2.5 antisera, kindly supplied by Dr. J. G. Ray, Transplantation and Immunology Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md. were used to differentiate between $H\text{-}2^d$ and $H\text{-}2^z$ haplotypes.

Monitoring Renal Dysfunction

MEASUREMENT OF PROTEINURIA. The onset of renal disease was monitored by fortnightly testing for proteinuria by using the method described previously (2). Briefly, 10-µl samples of urine were spotted onto strips of chromatography paper, fixed in ethanol, and stained with bromophenol blue. The degree of proteinuria in each mouse was assessed by visually comparing the color intensity of the urine spots with that of spots of bovine serum albumin standards (3,000, 1,000, 333, 111, and 37 mg/100 ml that had been similarly treated.

MEASUREMENT OF RENAL CLEARANCE. Detection of heavy proteinuria (333 mg/100 ml) was followed by the assessment of glomerular function with a single injection ^{51}Cr-EDTA clearance method (2). Studies on healthy 12-wk old mice established the normal half-value time as 14.0 ± 3.1 min. A half-value time of greater than twice the normal value was arbitrarily chosen as indicative of severe renal impairment (3).

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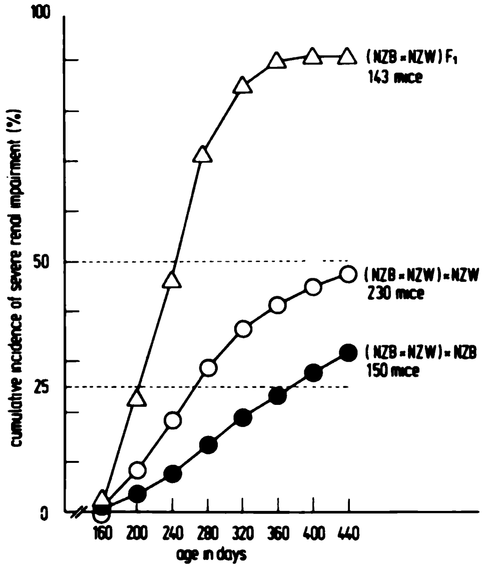

FIG. 1. A comparison of the cumulative incidence of severe renal impairment in NZB × NZW mice and in the reciprocal back-crosses.

Results

Incidence of Renal Disease in NZB, NZW, and NZB × NZW Mice. By using the criteria outlined above we observed 6 of 75 NZB mice (8%) and 1 of 40 NZW mice to have severe renal disease before the age of 440 days. In striking contrast, 130 of 143 (NZB × NZW)F_1 mice (92%) had severe renal disease before 440 days. We observed a continuing occurrence of renal disease in older NZB mice, the incidence reaching 16% by 540 days.

Incidence of Renal Disease in (NZB × NZW)F_1 × NZB Backcross Mice. Fig. 1 shows that the incidence of renal disease in the group of 150 (NZB × NZW) × NZB backcross mice reached 32.7% by 440 days. As we have observed a small, but significant, incidence of early renal disease in NZB mice we consider it more likely that this represents an over-estimate of 25% than an under-estimate of 50% incidence of early renal disease in this backcross. This view is strengthened by the observation that there was a marked difference in the distribution of ages of onset of renal disease in the mice of this backcross as compared to the NZB × NZW animals, with some of the backcross mice showing a tendency towards later onset of renal disease as occurs in the NZB strain. Thus 14 of 49 backcross mice developed renal disease when older than 360 days as compared to 2 of 130 NZB × NZW mice ($\chi^2 = 28.7, P < 0.001$).

These data, together with those of our earlier study (2), are therefore compatible with the conclusion that the renal disease of the NZB × NZW hybrid is determined by three genes, all dominant or codominant, one contributed by the NZB strain and two, unlinked, contributed by the NZW strain. We have designated these three genes LN-1 (lupus nephritis-1),[1] LN-2, and LN-3, respectively (see Fig. 2).

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[1] *Abbreviation used in this paper:* LN, lupus nephritis.

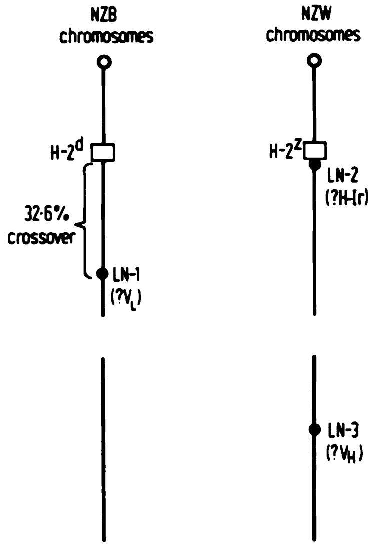

FIG. 2. Three genes for lupus nephritis (LN-1, LN-2, and LN-3), all dominant, in NZB × NZW mice. LN-1, LN-2, and LN-3 could represent clusters of closely linked genes rather than single loci.

TABLE I

Segregation of Renal Disease and H-2 Type in (NZB × NZW)F_1 × NZB Back-cross Mice

	Histocompatibility type		Total
	H-2^d/H-2^z	*H-2^d/H-2^d*	
Mice with renal disease	43	6	49
Mice without renal disease	35	66	101
Total	78	72	150

Linkage between the H-2 Complex and One of the NZW Renal Disease Genes. The mice of the (NZB × NZW) × NZB backcross would be expected to be either homozygous (H-2^d/H-2^d) or heterozygous (H-2^d/H-2^z) with respect to the *H-2* complex (NZBs are H-2^d, NZWs are H-2^z) (4). Table 1 shows that, as expected, the 150 mice were nearly equally divided between the two genotypes. However, a great preponderance of mice which developed renal disease were heterozygous. This difference is highly significant ($\chi^2 = 35.2$, $P < 0.001$) and indicates that one of the genes contributed by the NZW strain is linked to the *H-2* complex. The occurrence of renal disease in H-2^d/H-2^d mice could indicate that there is a crossover frequency of approximately 8% between the renal disease gene and the *D* end of the *H-2* complex, the side on which the new gene lies being undetermined. However, as already indicated, a small proportion (8%) of NZB mice develop early severe renal disease and these are indistinguishable from NZB × NZW mice on the basis of the criteria used in this study. Therefore it seems more probable that the six homozygous mice showing renal disease are not the result of crossover and that this NZW renal disease gene, which we have designated LN-2, is very closely linked to the *H-2* complex as indicated in Fig. 2.

Incidence of Renal Disease in (NZB × NZW)F_1 × NZC Mice. NZC mice have not been found to show any incidence of autoimmune lupus nephritis (5), so the incidence of renal disease in the (NZB × NZW) × NZC out-cross was studied as an independent test for linkage between renal disease genes from the NZB and the NZW strains. If the gene contributed by the NZB strain is on the same chromosome as one from the NZW strain then renal disease would only occur if there was crossingover between them. We observed 10 of 190 mice (5.3%) of this out-cross to develop renal disease. This figure approximates the 7.5% incidence which one would expect if the renal disease were determined by two dominant unlinked genes (A and B) from one strain and one dominant gene (C) from the other strain, linked to gene A but separated from it by a crossover frequency of 30%.

Only 100 of the 190 mice were *H-2* typed before some mice died of renal failure. However all six of the mice which developed renal disease and which had been typed were $H\text{-}2^{d/z}$, (NZC mice are $H\text{-}2^{d}$) (6) which suggests that (one of) the gene(s) from the NZW is closely linked to the *H-2* complex. Thus the data obtained from this out-cross provide independent confirmation of the data obtained from the reciprocal backcrosses.

Discussion

In cross-breeding studies of the NZB mice with other inbred New Zealand strains, Helyer and Howie (7) observed that NZB × New Zealand Yellow F_1 hybrids showed only a very low incidence of hemolytic anemia and positive Coombs tests (4%) but died prematurely with renal lesions similar to those seen in lupus nephritis. Subsequently Helyer and Howie (8) reported that NZB × NZW mice die even more prematurely from a similar lupus nephritis. Positive lupus erythematosus cell tests were observed in both hybrids (8). It was shown by fluorescence techniques that NZB × NZW hybids have accumulations of immunoglobulins in the glomeruli (9, 10), together with complement (10), and elution studies have shown that the immunoglobulins have specificity for nuclear antigens in accord with the concept that the renal lesions are caused by immune complexes of nuclear antigens and their complement-fixing autoantibodies which become trapped in the glomeruli (10).

NZB and NZW mice from the Otago colonies do not show the early renal failure characteristic of the NZB × NZW hybrids. Howie and Helyer (1) found no heavy proteinuria in 270 virgin female NZB mice at 1 yr of age and we observed an incidence of only 4% in a similar group of 75 NZB mice aged 1 yr. Similarly we have observed only a very low incidence (2.5%) of early renal disease in 40 NZW mice.

It appears that renal disease and/or antibodies directed against nuclear components are found much more commonly in colonies of NZB and NZW mice elsewhere in the world. Thus reports on anti-nuclear antibody incidence in NZB mice have varied from almost 0 to 100% (1, 11-16). It is highly relevant that many of the authors reporting a high incidence of anti-nuclear antibody (ANA) in NZB mice have also observed considerable frequencies of ANA in unrelated strains, suggesting that environmental factors could be largely responsible, as indicated by the studies of Barnes and Tuffrey (17) and Shulman et al. (18).

Dixon et al. (19) demonstrated that virus infections could cause nephritis in normal mouse strains as a result of deposition of viral-anti-viral-antibody complexes in glomeruli. The presence of C-type particles indicative of murine leukemia virus in NZB mice appears to be widely accepted as a normal attribute of the strain. However, C-type particles have never been observed in mice from the Otago colony despite extensive electron microscope studies (20) and serological testing of serum samples from the Otago colonies has failed to reveal the presence of active murine leukemia virus, T. Maguire, personal communication. Differences in viral infestations could well explain many of the discrepancies in the literature on the NZB mouse and its hybrids.

It now seems clear that the autoimmunity is not due to virus infection in that it cannot be transmitted by cell-free filtrates (21, 22) or enhanced by immune suppression (23, 24). On the other hand, the lymphoid tumours which occur with widely varying frequency in different NZB colonies (21) do appear to be caused by oncogenic viruses in that their frequency is greatly increased by immune suppression (23, 25) and they appear to have been transmitted by cell-free filtrates (26) as well as by living cells (27, 28). It seems likely that these lymphoid tumours are unrelated to the autoimmunity (23).

Our previous study (2) showed a 50% incidence of severe early renal disease in (NZB × NZW) × NZW backcross animals, indicating that the NZB strain contributes a single gene, or cluster of closely-linked genes, to the condition. This gene was found to be loosely linked to the *H-2* complex (see Fig. 2). The present study, showing a 25% incidence of early renal disease in (NZB × NZW) × NZB backcross animals, indicates that the NZW strain contributes two unlinked genes. However, additional genes, common to both the NZB and the NZW strains, could also be involved in the lupus nephritis. One of the NZW genes was found to be closely linked to the *H-2* complex (Fig. 2) and could be a histocompatibility-linked immune response (*H-Ir*) gene (29). Of interest in this regard is the observation of Lambert and Dixon (30) that NZW mice are high responders to heat denatured DNA coupled to methylated bovine serum albumin, and that high responder status is transferred in dominant fashion to the NZB × NZW hybrid (NZBs are low responders to DNA).

All three of the genes whose effect we have observed must be dominant or codominant in that their effect is expressed in the heterozygous state. From consideration of the features and pattern of inheritance of thyroid and other human autoimmune diseases one of us (31) has postulated that the genetic basis of inherited autoimmune disease lies in the specificity of V genes, the structural genes which code for the variable portions of the light and heavy polypeptide chains which combine to form antibody molecules (32). We consider that the genes LN-1 and LN-3 are most likely to be immunoglobulin V genes in accord with this concept. It is possible that LN-1 is a light chain V gene and LN-3 a heavy chain V gene which, in combination, code for an autoantibody in the NZB × NZW hybrid (Fig. 2). The crossover frequency between LN-1 and the *D* end of H-2, 32.6% (2), is very close to that (31.4%) for a gene observed by Lotzova and Cudkowicz (33) to control resistance to bone marrow grafts in mice. These two genes could be members of a single cluster of V genes.

A widely-held hypothesis proposes that autoimmune disease arises as a result of an age-dependent loss of suppressor T cells, which are supposed to prevent production of autoantibodies in normal animals (34, 35). It has been claimed that suppression or prevention of autoantibody formation can be achieved in mice of strains which develop autoimmunity by injecting thymocytes from young syngeneic donors (36, 38). However, we have been unable to confirm these findings in NZB and NZB × NZW mice.[2] Furthermore, it is difficult to see how such a proposal could be compatible with present knowledge of the genetic basis of autoimmune diseases. The pattern of inheritance of human autoimmune diseases is dominant with incomplete penetrance and for thyrotoxicosis and diabetes there is evidence that at least two genes are involved, suggesting a basis in pairs of immunoglobulin V genes (31) and making an interesting parallel with the present data.

Summary

The occurrence of early severe lupus nephritis in (NZB × NZW)F_1 mice must depend on the action of at least two dominant or codominant genes (at least one gene from each parent) as neither of the inbred parental strains shows the disorder. Identifying affected animals by antemortem determinations of renal function, we have studied the incidence of the renal disease in 230 (NZB × NZW) × NZW backcross mice (an earlier study) and, in this study, in 150 (NZB × NZW) × NZB backcross mice. The data indicate that the NZB strain contributes only one gene and the NZW strain contributes two genes, or clusters of closely linked genes, to the renal disorder of the F_1 hybrid. One of the NZW genes was found to be linked to the *H-2* complex. All three genes must be dominant or codominant, as their effect is expressed in the heterozygous state.

We are grateful to Ms. Fiona McKenzie, Ms. Judith Burborough, and Mr. W. S. Cague for excellent technical assistance.

Received for publication 21 November 1977.

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CYTOTOXIC T-CELL RESPONSES SHOW MORE RESTRICTED SPECIFICITY FOR SELF THAN FOR NON-SELF *H-2D*-CODED ANTIGENS

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Responses of murine, thymus-derived, cytotoxic lymphocytes (Tc cells)[1] against foreign antigens displayed by various modified-self or H-2 compatible cells, exhibit H-2 restriction (1-3). Thus, Tc cells specifically recognize both foreign antigen and molecules coded in the *K* or *D* regions of the *H-2* complex (4, 5).

In the case of virus-infected self cells, it is apparent that various virus-specified proteins may carry the foreign component essential to the antigenic pattern recognized by Tc cells, thus conferring virus-specificity (6-10). The contribution of *H-2K* and *H-2D* regions has been clarified by the use of *H-2* mutants. Biological and biochemical studies of a series of mutants in the *H-2K* region of the $H\text{-}2^b$ haplotype have demonstrated first that the classical, serologically-defined H-2 alloantigen molecule bearing private specificities is essential for Tc cell recognition of infected-self cells (11-15), and second, that the specificity of self H-2K antigen recognition by Tc cells is exquisite (11-13, 16). For example, mutant and wild-type H-2K molecules which are qualitatively indistinguishable by serological criteria (17, 18) and which crossreact markedly when recognized by alloreactive Tc cells (19-21), do not crossreact when recognized by Tc cells specific for virus-plus-self (11-13, 16).

Since high specificity of self recognition is an essential characteristic of the H-2 restriction phenomenon, it was investigated further with respect to the specificity of recognition of *H-2D*-coded molecules.

The *D* region of the $H\text{-}2^d$ haplotype apparently codes for two different 45,000 dalton antigen molecules designated D and D′ by Hansen et al. (22). D carries the serologically-defined private specificity H-2.4 and D′ carries certain public specificities reactive to anti-H-2.28 sera (22, 23). The experiments reported here employed the BALB/c-$H\text{-}2^{db}$ mutation, a loss mutation in the $H\text{-}2D^d$ region which results in a lack of expression on cell surface membranes of D′, while D is expressed normally (22, 23). Both D′ and D are expressed on the cell membranes of wild-type BALB/c mice. The results show that Tc cells specific for $H\text{-}2D^d$ coded antigens plus viral or minor histocompatibility (H) antigens on syngeneic

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[1] *Abbreviations used in this paper*: D, $H\text{-}2D^d$-coded antigen molecule bearing private specificity H-2.4; D′, $H\text{-}2D^d$-coded antigen molecule bearing public specificity H-2.28; MLR, mixed lymphocyte reaction; PFU, plaque-forming units; T cell, thymus-derived lymphocyte; T_c cell, cytotoxic T cell.

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or H-2 compatible cells, respectively, recognize D but do not detectably recognize D′. On the other hand, BALB/c-H-2^{db} Tc cells respond in mixed lymphocyte reactions (MLR) to the D′ present on BALB/c cells, and third party responder strains in MLR against BALB/c also produce Tc cells that recognize D′.

Materials and Methods

Animals. A/J, A.TH, A.TL, BALB/c, BALB/c-H-2^{b}, BALB/c-H-2^{db}, CBA/H, C3H.OH, C3H.OL, C57BL/6, DBA/1, and SJL/J mice were bred in conventional rooms and both sexes were used at 2–3 mo of age.

Immunization. Mice were immunized with ectromelia virus by intravenous (i.v.) injection of 10^4 plaque-forming units (PFU) of the attenuated Hampstead egg strain (24). Virus-specific memory T cells were obtained from the spleens 3 wk or more postimmunization (25).

Generation of Tc Cells in Vitro. The methods for secondary virus-specific responses, secondary responses to minor H antigens, and primary one-way MLR have been given in detail elsewhere (3, 25–27).

Briefly, secondary virus-specific T_c cells were generated by stimulating splenic memory T cells by coculturing them with syngeneic, ectromelia virus-infected splenic stimulator cells for 5 days at 39°C (nonpermissive for ectromelia replication) at a responder-stimulator ratio of 10:1 (25, 26).

Primary MLR utilized splenic responder cells and γ-irradiated, allogeneic, splenic stimulator cells cultured at 37°C for 5 days at a responder:stimulator ratio of 4:1 (27).

For the response to minor H antigens the method used was similar to that of Bevan (3). Responder mice were primed by i.v. injection of 10^7 spleen cells from appropriate H-2-compatible donor strains. After 2–4 wk spleen cells from the primed mice were stimulated a second time in vitro by coculturing for 5 days at 37°C with irradiated splenic stimulator cells, and Tc cell activity against minor H antigens was then assayed on macrophage targets.

Cytotoxicity Assay with Macrophage Target Cells. The ^{51}Cr release method using macrophages has been described fully elsewhere (13). Statistical significance was determined by Student's *t* test.

Results

Specificity of Tc Cell Recognition of Viral or Minor H Antigens in Association with Self H-2D-Coded Antigen. Previous results showed that ectromelia virus-infected target cells from BALB/c (H-2^d) and BALB/c-H-2^{db} mice were lysed to a similar extent in a 16-h assay by Tc cells generated during primary viral infection in vivo of various mouse strains which shared only the *D* region of the H-2^d haplotype with the target cells (15). Since BALB/c-H-2^{db} cells do not express the D′ molecule on their surface membranes whereas BALB/c cells do (22) these original results implied that D′ was not detectably recognized in association with virus-specific antigens by these Tc cells. The present studies have confirmed and extended this conclusion. Tc cells from A.TH mice (K^s, D^d) generated in a secondary response in vitro lysed infected BALB/c and BALB/c-H-2^{db} macrophage targets to the same extent and with similar kinetics in short-term assays (Table I).

Similar results (data not shown) have also been obtained with Sendai virus, a paramyxovirus quite different biologically and antigenically from ectromelia virus (a poxvirus).

Since the recognition by Tc cells of minor H antigens on H-2 compatible cells is H-2 restricted in a manner analogous to viral systems, the involvement or otherwise of D′ was investigated in two examples of Tc cell responses to minor H antigens.

In the first example B10.A (K^k, D^d) mice were injected i.v. with A/J (K^k, D^d) cells and then their primed spleen cells were stimulated in vitro with A/J cells

TABLE I

Similar Lysis of Infected BALB/c-H-2^{db} and BALB/c Macrophage Targets by Virus-Immune A.TH T_c Cells Specific for H-2D-Region-Coded Antigens

Donors of virus-immune T_c cells*			Assay time h	Specific lysis of targets‡			
Strain	H-2K	H-2D		BALB/c-H-2^{db}		BALB/c	
				Infected	Uninfected	Infected	Uninfected
A.TH	s	d	4	9.1 ± 1.6	0	15.6 ± 1.7	1.7 ± 0.4
C3H.OL‡§	d	k	4	29.4 ± 4.8	1.9 ± 0.7	31.8 ± 4.3	3.4 ± 0.5
A.TH	s	d	6	25.0 ± 1.2	3.3 ± 1.3	30.5 ± 4.4	1.3 ± 0.9
C3H.OL	d	k	6	38.5 ± 3.9	2.7 ± 0.3	36.0 ± 4.7	3.0 ± 1.3
A.TH	s	d	9.5	40.9 ± 0.8	7.2 ± 1.3	39.4 ± 3.8	9.8 ± 1.8
C3H.OL	d	k	9.5	52.5 ± 6.4	7.1 ± 1.3	46.5 ± 5.9	7.2 ± 2.5

* From secondary responses in vitro. Killer:target ratio was 10:1.

‡ Data given are means of triplicates ± SE of mean with spontaneous release subtracted.

§ C3H.OL T_c cells were used as controls to demonstrate that the intrinsic susceptibility to lysis of infected BALB/c-*H-2*^{db} and BALB/c targets was similar in the case where recognition was via *H-2K*-coded antigen (unaltered by the BALB/c-*H-2*^{db} mutation).

TABLE II

T_c Cells Do Not Detectably Recognize D′ in Association with Minor H Antigens on H-2 Compatible Cells

Exp.	T_c cells*	Killer target ratio	Percent specific lysis of macrophage targets‡					
			A/J (k kkdd d)§	A.TH (s ssss d)	BALB/c (d dddd d)	BALB/c-*H-2*^{db} (d dddd "db")	BALB/c-*H-2*^{b} (b bbbb b)	C3H.OH (d dddd k)
1.	B10.A (k kkdd d)	2.5:1	65.3 ± 1.4‖	45.7 ± 2.3	49.0 ± 1.5	45.8 ± 4.2	2.4 ± 0.6	3.1 ± 0.1
	Anti-A/J (k kkdd d)	0.8:1	40.2 ± 2.0‖	24.5 ± 0.7	14.1 ± 0.9	11.5 ± 1.6	1.3 ± 0.4	0.8 ± 0.4
			A.TL (s kkkk d)	C3H.OL (d dddk k)	CBA/H (k kkkk k)	SJL/J (s ssss s)		
2.	DBA/2 (d dddd d)	7.5:1	33.5 ± 1.0	59.6 ± 2.3	9.8 ± 2.0	10.8 ± 1.0		
	Anti-BALB/c	2.5:1	12.6 ± 0.8	33.1 ± 1.2	4.5 ± 2.2	8.7 ± 1.9		
	DBA/2 (d dddd d)	7.5:1	32.4 ± 3.8	60.0 ± 2.0	11.7 ± 4.0	11.8 ± 1		
	Anti-BALB/c-*H-2*^{db}	2.5:1	13.1 ± 2.2	34.8 ± 2.3	2.5 ± 1.0	4.7 ± 2.5		

* As for Table I.

‡ As for Table I.

§ H-2 maps refer to *K*, *I-A*, *I-B*, *I-C*, *S*, and *D* regions.

‖ Significantly more lysis than A.TH targets at the same killer target ratio ($P < 0.001$).

and the resulting Tc cells, directed against minor H antigens in association with K^k or D^d-coded molecules, were assayed against various targets. A/J (K^k, D^d) targets were lysed significantly more than A.TH (K^s, D^d) targets, presumably because only Tc cells recognizing minor H plus D^d coded determinants killed A.TH targets, whereas additional Tc cells recognizing minor H plus K^k-coded determinants would kill A/J targets (Table II, exp. 1). BALB/c and BALB/c-*H-2*^{db} targets were also lysed, presumably by Tc cells recognizing minor H antigens shared by BALB/c and A/J and associated with D^d-coded determinants. Lysis of BALB/c and BALB/c-*H-2*^{db} targets was similar; assuming the targets had the same intrinsic susceptibility, this result implies that D′ (lacking in BALB/c-*H-2*^{db}) was not recognized in association with a significant number

TABLE III

BALB/c-H-2^{db} *Anti-BALB/c* T_c *Cells Recognize Antigenic Patterns Dependent upon the H-2D*^{d} *Region*

T_c cells	Killer: target ratio	Percent specific ^{51}Cr release from macrophage targets*				
		BALB/c-*H-2*^{db} (d dddd "db")‡	BALB/c (d dddd d)	A.TH (s ssss d)	C3H.OH (d dddd k)	SJL/J (s ssss s)
BALB/c-*H-2*^{db}	17:1	1.6 ± 1.9	40.6 ± 4.6	41.6 ± 0.8	5.9 ± 1.6	6.0 ± 2.1
Anti-BALB/c	5.6:1	1.0 ± 2.3	32.8 ± 5.7	28.5 ± 2.2	5.3 ± 2.0	7.6 ± 1.3

* See ‡, Table I.

‡ See §, Table II.

of minor H antigens by the B10.A Tc cells that responded to H-2-compatible A/J cells.

In a second approach to this question, spleen cells from DBA/2 (H-2^{d}) mice previously primed with either BALB/c or BALB/c-*H-2*^{db} cells, were stimulated a second time in vitro with irradiated BALB/c or BALB/c-*H-2*^{db} cells, respectively. Tc cell activity was then assayed against C3H.OL (K^{d}, D^{k}) and A.TL (K^{s}, D^{d}) macrophage targets that would display minor H antigens shared with BALB/c in association with either K^{d}-coded (C3H.OL) or D^{d}-coded (A.TL) determinants. At a given killer:target ratio, C3H.OL targets and nonspecific controls (CBA/H and SJL/J) were lysed to a similar extent by the two Tc cell populations resulting from stimulation by either BALB/c or BALB/c-*H-2*^{db} cells (Table II, exp. 2), thus indicating that the strength of the two responses was similar, and that valid comparison of lysis of A.TL targets was possible. A.TL targets were lysed to a similar extent, regardless of whether stimulation was from BALB/c or BALB/c-*H-2*^{db} cells (Table II, exp. 2). If DBA/2 anti-BALB/c Tc cells recognized significant numbers of minor H antigens in association with D′, then they should have lysed A.TL targets to a greater extent than DBA/2-anti-BALB/c-*H-2*^{db} Tc cells, provided that the responses were approximately equal in other respects as indicated by the controls.

Taken together, these experiments suggest the general rule that D′ is not recognized in Tc cell responses against self *H-2D*^{d}-coded antigens plus foreign antigens. Instead, the D molecule is apparently recognized.

Recognition of D′ in Tc Cell Responses against Non-Self or H-2-Incompatible Cells. Primary MLR of BALB-c-*H-2*^{db} anti-BALB/c (H-2^{d}) gave strong Tc cell responses roughly comparable to other combinations with differences only in *K* or *D* regions (Tables III and IV). A.TH targets were lysed as much as BALB/c targets (Table III) though they share only the D^{d} region with BALB/c, whereas C3H.OH targets, which share all of the *H-2* gene complex with BALB/c, except for the *D* region, were lysed no more than SJL (H-2^{s}) nonspecific control targets (Table III). These data thus confirm other serological and cell-mediated results that map the BALB/c-*H-2*^{db} mutation in the *D* region (23).

Serologically, D′ does not bear private H-2 specificities, but reacts with antibodies present in antisera raised against the H-2.28 family of public H-2 specificities (22, 23). Tc cell responses against D′ also displayed marked crossreactivity; thus targets from other mouse strains bearing the H-2.28 family

TABLE IV

BALB/c-H-2^{db} Anti-BALB/c T$_c$ Cells Recognize Mainly "Public" Rather Than "Private" H-2 Determinants

Target cells				Percent specific lysis* with BALB/c-*H-2*^{db} anti-BALB/c T$_c$ cells at killer:target ratio	
Strain	H-2	Serologically defined public specificities of the "H-2.28 family"	Titer with BALB/c-*H-2*^{db} anti-BALB/c serum‡	10:1	3:1
BALB/c	d	27, 28, 29	32	62.8 ± 0.6	46.6 ± 2.7
DBA/1	q	27, 28, 29	64	62.4 ± 2.9	28.4 ± 1.5
C57BL/6	b	27, 28, 29	32	48.0 ± 4.1	25.1 ± 3.5
SJL/J	s	28	0	18.3 ± 3.6	3.6 ± 1.4
(DBA/1 × BALB/c-*H-2*^{db})F$_1$				42.5 ± 3.4	19.9 ± 2.9
(C57BL/6 × BALB/c-*H-2*^{db})$_1$				26.0 ± 2.9	9.2 ± 3.6

* See ‡, Table I.

‡ Data from McKenzie et al. (23).

of specificities were also lysed significantly by BALB/c-*H-2*^{db} anti-BALB/c Tc cells (Table IV). The Tc cell crossreactivities were, however, not as prominent as in the serology, particularly in the case of C57BL/6 (H-2^b). This may mean that Tc cells recognize different determinants on D′ from those defined serologically, as previously suggested by results using mutants in the *K* region of the *H-2*^{b} haplotype (18–20), or that Tc cell receptor affinity is lower than that of most immunoglobulins (16, 21).

The marked Tc cell crossreactivity seen in Table IV argues against the idea that the response to D′ is H-2 restricted i.e., that determinant(s) on the D′ molecule are recognized mainly or exclusively in association with determinant(s) unique to the *H-2*^{d} haplotype. This possibility was tested further by the use of targets from F$_1$ hybrids between DBA/1 or C57BL/6 and BALB/c-*H-2*^{db} (Table IV). The BALB/c-*H-2*^{db} genome codes for all other detectable H-2^d determinants other than those on D′. Thus, if the BALB/c-*H-2*^{db} anti-D′ responses were H-2 restricted, the F$_1$ targets should be killed more efficiently than DBA/1 or C57BL targets. However, the converse was observed; they were lysed less (Table IV), thus confirming the lack of H-2 restriction.

Is there a Tc cell response to D′ in MLR in which there are concurrent responses to other H-2K or H-2D antigen molecules? C57BL/6 (H-2^b) anti-BALB/c (H-2^d) T cells lysed A.TL (*K*^{s}, *D*^{d}) or A.TFR2 (*K*^{f}, *D*^{d}) targets significantly more than did C57BL/6 anti-BALB/c-*H-2*^{db} Tc cells, whereas C3H.OL (*K*^{d}, *D*^{k}) targets were lysed equally by both Tc cell populations (Table V, exp. 1). These data suggest that more C57BL/6 Tc cells reactive to *H-2D*-coded antigens were stimulated by BALB/c than by BALB/c-*H-2*^{db} cells. A response to determinants on D′ (other than those shared by C57BL/6 and BALB/c) would account for this.

TABLE V

Evidence for a T_c Cell Response to D′ in Allogenic MLR

Exp.	T_c cells	Percent specific lysis of macrophage targets*				
		A.TL (s kkkk d)‡	A.TFR2 (f fffs d)	C3H.OL (d dddk k)	CBA/H (k kkkk k)	SJL/J (s ssss s)
1.	C57BL/6 Anti-BALB/c (b bbbb b) (d dddd d)	70.8 ± 0.9	68.3 ± 0.3	51.3 ± 1.4	18.3 ± 3.4	15.5 ± 0.6
	C57BL/6 anti-BALB/c-*H-2*^{dm} (b bbbb b) (d dddd "db")	56.9 ± 1.8§	56.1 ± 3.1§	51.8 ± 4.5	15.0 ± 3.4	15.2 ± 3.6
2.	CBA/H Anti-A.TFR5 (k kkkk k) (f fffk d)	CBA/H (k kkkk k)	A.TH (s ssss d)	DBA/1 (q qqqq q)		
		10.6 ± 0.9	86.9 ± 1.8	49.4 ± 3.2		

* See ‡, Table I; killer:target ratio was 3:1.

‡ See §, Table II.

§ Significantly less lysis than that caused by C57BL/6 anti-BALB/c T_c cells on the same target cells ($P < 0.02$).

Furthermore, CBA/H (*H-2*^{k}) anti-A.TFR5 (K^f, D^d), Tc cells which lysed A.TH (K^s, D^d) targets, crossreacted markedly on DBA/1 (*H-2*^{q}) targets that share determinants with D′ (Table V, exp. 2). Thus a Tc cell response to determinants on D′ probably occurred in both these MLR. Since other workers have reported similar crossreactivity involving the H-2.28 family of public determinants in MLR-generated Tc cells (28), these data suggest the general rule that D′ is recognized in Tc cell responses against *H-2D*^{d}-coded antigens where such antigens are non-self.

Discussion

Highly specific recognition of self H-2 antigens by Tc cells reactive to virus-infected self cells, or reactive to minor H antigens on uninfected H-2-compatible cells, is essential to the phenomenon of H-2 restriction. If such Tc cells were crossreactive or recognized determinants which are shared by different H-2K or H-2D antigens (i.e., public determinants), then H-2 restriction would of course, not be observed.

The present experiments illustrate this point with respect to recognition by Tc cells of the two antigens D and D′ coded in the *H-2D*^{d} region. D carries private, and D′ carries public determinants (22, 23). Apparently, Tc cells reactive to virus-infected self cells, or Tc cells reactive to minor H antigens plus *H-2D*^{d}-coded antigens on H-2-compatible cells recognize determinant(s) on the self D molecule together with physically associated non-self antigen(s). They do not recognize D′ in detectable numbers. Since similar results were obtained with two very different viruses, ectromelia (a poxvirus) and Sendai (a paramyxovirus), and with two different minor H antigen systems, it seems reasonable to propose that these data reflect the general rule that recognition of self *H-2D*^{d}-coded antigens by H-2 restricted Tc cells involves antigenic determinant(s) on the D molecule, not on D′.

One possible reason for this might be that D′ does not associate physically with other antigens in cell membranes. Available data suggest that this is not the case. First, there is evidence that D and D′ co-cap (29). Second, antisera specific for D or for D′ can block virus-specific Tc cell-mediated lysis of virus-infected targets, thus indicating that both molecules are physically close to virus-specific determinants in infected cell surfaces (15). Other reasons must, therefore, be considered. For example, the dictionary of Tc cell antigen-receptors employed in responses against infected self cells may not encompass D′ determinant(s). This possibility is discussed further below.

In contrast to the case of recognition of infected self, D′ *is* recognized by Tc cells which have developed in its absence. Thus, Tc cells from BALB/c-*H-2*^{db} (D′ negative) mice respond strongly in MLR to the D′ antigen of BALB/c cells. D′ is apparently recognized as a major alloantigen in its own right, rather than as a minor H antigen associated with D, i.e., as an example of "H-2 restriction". The evidence for this is threefold. First, BALB/c-*H-2*^{db} anti-BALB/c Tc cells crossreact markedly on DBA/1 (*H-2*^{q}) and C57BL/6 (*H-2*^{b}) target cells, both of which display the "H-2.28 family" of public specificities but do not share the D molecule coded in the *H-2*^{d} haplotype of BALB/c which would be required for H-2 restricted recognition. Second, F_1 hybrid targets of the type (DBA/1 × BALB/c *H-2*^{db}) or (C57BL/6 × BALB/c-*H-2*^{db}) are killed less by such crossreactive Tc cells than DBA/1 or C57BL/6 targets. If the rules of H-2 restriction applied to this response, F_1 targets should be killed more than targets from DBA/1 or C57BL/6, since the D molecule is contributed by the BALB/c-*H-2*^{db} haplotype of the F_1 hybrids. Third, Tc cells that recognize D′ appear to comprise a significant part of the Tc cell populations that respond to *H-2D*^{d}-coded antigens in other MLR, e.g., C57BL/6 anti-BALB/c, and CBA/H anti-A.TFR5.

It would seem, therefore, that with respect to Tc cell recognition D′ is in a category similar to *I* region-coded antigens. Such antigens can be recognized by some clones in allogeneic Tc cell populations, (30–32), albeit at a lower frequency that H-2K or H-2D antigens, but they are not directly involved in the recognition by Tc cells of virus-infected self cells, or H-2 compatible cells bearing non-self minor H antigens (3–5).

Another interesting aspect of the present results is their implication for mechanisms by which the antigen-receptor dictionary of the Tc cell pool is governed, presumably in the thymus during the differentiation of stem cells into mature, antigen-sensitive precursor Tc cells as illustrated by recent results of Bevan (33) and Zinkernagel et al. (34). This differentiation process presents an intriguing problem. On the one hand, Tc cell receptors must be diverse enough to recognize any potential viral pathogen that poses a threat to host survival. On the other hand, it seems that an H-2K or H-2D molecule is almost invariably recognized simultaneously with the foreign antigen, i.e., there is a restricted repertoire of determinants characteristic of *H-2K*- or *H-2D*-coded molecules that must also be recognized at the induction and effector phases of Tc cell operation.

Results from chimeric mice (33, 34) grafted with allogeneic thymic epithelium after removal of their own thymus (34), have fostered speculation that the thymic influence on Tc cell differentiation incorporates a selection or screening process in which progenitors are selected for further maturation by their capacity to recognize the *H-2K*- or *H-2D*-coded determinants displayed by self thymic epithelial cells (34, 35). If this is the case, why aren't Tc cells reactive to self D′ selected? One trivial explanation might be that D′ is not expressed on thymic epithelial cell surface membranes. This is testable with available antisera. Alternatively, the screening process in the thymus may be more complex, and may permit only certain categories of self H-2 recognition to maintain self tolerance (36–39). Further investigation of factors controlling T-cell differentiation in the thymus sould resolve this question.

Summary

The specificity of recognition of H-2 antigens by various subsets of Tc cells was investigated with respect to the two separate molecules known to be coded in the *H-2D*^{d} region (*a*) D which carries the private specificity H-2.4 and (*b*) D′ which carries the public specificity H-2.28. BALB/c-*H-2*^{db} mutant mice express D but not D′ on their cell surfaces, whereas wild-type BALB/c mice express both D and D′. H-2 restricted Tc cells specific for viral-plus-*H-2D*^{d}-coded antigens on infected self cells, or minor H-plus-*H-2D*^{d}-coded antigens on H-2-compatible cells apparently recognize D, but do *not* detectably recognize D′. In contrast, BALB/c-*H-2*^{db} anti-BALB/c Tc cell responses do recognize D′ (the only known antigen which is not shared by mutant and wild-type); furthermore, D′ is also detectably recognized by a significant proportion of the Tc cells that respond in MLR to *H-2D*^{d}-coded antigens. In these latter responses, D′ was recognized separately from D, i.e., the response was not "H-2 restricted". These results indicate that H-2 restricted Tc cell responses to modified-self cells are more specific for self H-2D^d-coded antigens then are allogeneic Tc cell responses directed at the same antigens, in that haplotype-unique (private) specificity recognition (of the D molecule) exclusively occurs only in the former, not the latter case. The implications of this specificity of H-2 restricted responses for possible processes of somatic selection of anti-self recognition structures on progenitor Tc cells are briefly discussed.

We thank Marion Andrew for excellent technical assistance.

Received for publication 29 December 1977.

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HUMAN IMMUNE RESPONSES TO HAPTEN-CONJUGATED CELLS

I. Primary and Secondary Proliferative Responses in Vitro*

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The association between the human major histocompatibility complex (MHC)[1] and a variety of diseases has been well established in the last few years (1, 2). In contrast with the mouse, however, in which the availability of *H-2* recombinant strains has permitted rapid progress, relatively little is known about the functional role of MHC genes in human immune responses. In the mouse, genes within specific regions of the *H-2* complex determine susceptibility to certain diseases (3), control immune responses to numerous antigens (4), and determine proliferative and cytotoxic immune responses of T cells (5-8). Thus, T-cell-mediated cytotoxicity against virus-infected (5) or hapten-modified cells (6) and against minor histocompatibility locus antigens (7) requires syngenicity between target and original stimulator cells with respect to *K* or *D* regions. On the other hand, proliferative responses to protein antigens are facilitated by *I* region homology between interacting macrophages and lymphocytes in both guinea pigs and mice (8, 9). Secondary proliferative responses to hapten-modified cells may also be facilitated by homology within *H-2* (10).

Recently Newman et al. (11) have reported that primed human lymphoid cells respond to hapten-conjugated lymphoid cells in vitro. Using a similar system, the present report describes development of a human model to study both primary and secondary proliferative responses to trinitrophenylated human cells. Moreover, the data connote a role for the human MHC in T-cell recognitive processes. Human lymphocytes primed to hapten-modified autologous peripheral blood mononuclear cells gave substantially greater proliferative responses when restimulated with autologous than with allogeneic hapten-conjugated cells. HLA-A and HLA-B locus antigens do not appear to participate in such restimulation, whereas B-cell typing studies strongly suggest a role for gene products of the HLA-D region in definition of functional immunogenic units.

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* Supported by National Institutes of Health grants AI 13810 and RR 00350. Computational assistance was provided by the CLINFO Project, National Institutes of Health Contract NO1-RR-5-2118.

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[1] *Abbreviations used in this paper:* HBSS, Hanks' balanced salt solution; MHC, major histocompatibility complex; MLC, mixed leukocyte reaction; PBMCs, peripheral blood mononuclear cells; TNP, trinitrophenyl.

Materials and Methods

Peripheral Blood Mononuclear Cells (PBMCs). Heparinized venous blood was obtained from healthy human subjects and PBMCs were isolated by flotation on sodium diatrizoate-Ficoll cushions (Isolymph; Teva, Ltd., Jerusalem, Israel) by previously described techniques (12). Cells were washed twice with Hanks' balanced salt solution (HBSS; Microbiological Associates, Walkersville, Md.) before hapten conjugation or cell culture. Cells obtained in this fashion contain approximately 80% lymphocytes and 20% monocytes. An average of 70% of these cells were classified as T cells on the basis of E-rosetting with sheep erythrocytes.

Trinitrophenyl (TNP)-Conjugated Cells. Hapten-modified PBMCs were prepared by modifications of the technique described by Shearer for murine cells (13). Briefly, 5-25 × 10^6 cells/ml were incubated 12 min at 37°C with 2 mM trinitrobenzene sulfonic acid (Nutritional Biochemicals Corp., Cleveland, Ohio) in HBSS buffered with 50 mM Hepes (Microbiological Associates) and titrated to pH 7.8 with 1 N NaOH. Cells were washed three times with Hepes-buffered HBSS supplemented with 10 mg/ml glycylglycine (Grand Island Biological Co., Grand Island, N.Y.) and 5% heat-inactivated fetal calf serum (Grand Island Biological Co.) to remove all unreacted trinitrobenzene sulfonic acid.

Cell Culture Techniques. Cells were cultured in RPMI-1640 (Grand Island Biological Co.) supplemented with 20% AB positive heat-inactivated pooled human plasma, 2 mM L-glutamine (Microbiological Associates) and 50 μg/ml gentamicin (Schering Corp., Kenilworth, N.J.). Cultures were buffered to pH 7.2 with 25 mM Hepes and 1 N NaOH and were incubated in a 5% CO_2 atmosphere at 37°C.

For primary stimulation cells were cultured in either round-bottom microculture wells (IS-MRC-96; Linbro Scientific Inc., Becton, Dickinson & Co., New Haven, Conn.) or in round-bottom 17 × 100-mm tubes (2001; BioQuest, BBL, & Falcon Products, Cockeysville, Md.). In general, tubes were utilized in long-term cultures for ease in media replacement. Responder and stimulator cells were each cultured at a final concentration of 7.5 × 10^5 cells/ml in cultures of 0.2 ml (microculture wells) or 2 ml (tubes). For primary sensitization the cells were cocultured for 21-30 days. Primary proliferative responses were monitored from 3 to 25 days after culture initiation. Stimulator cells were treated with 500 or 4,000 rads ^{137}Cs (dose rate 6,000 rads/min) or with mitomycin C (Sigma Chemical Co., St. Louis, Mo., 50 μg/ml for 30 min at 37°C). Inactivated stimulator cells are designated throughout with a subscript x. Culture media were replaced with fresh media once each week.

For secondary restimulation, primed cells were harvested, washed once with medium, and cocultured in microculture wells at a final concentration of 7.5 × 10^5 viable cells/ml, with an equal concentration of fresh stimulator cells. Stimulator cells in secondary cultures were either TNP-conjugated or unconjugated, and in all experiments were inactivated with 4,000 rads γ-irradiation.

Proliferative responses were assessed by addition of 1.0 μCi of tritiated thymidine (sp act 2.0 Ci/mM; New England Nuclear Corp., Boston, Mass.) to cultures for the final 20 h of the incubation period.

HLA Typing. Typing for HLA-A and HLA-B locus antigens was by standard serologic techniques, employing the microcytotoxicity assay (14). All serologic procedures were performed by personnel of the Methodist Hospital tissue typing laboratory.

B-Cell Antigen Typing. B-cell antigens were detected by using the method of Ting et al. (15). Briefly, a B-cell-enriched population was prepared by rosetting PBMCs with neuraminidase-treated sheep erythrocytes over sodium diatrizoate-Ficoll cushions. The non-erythrocyte rosetting (interface fraction after centrifugation) B-cell-enriched population was then typed for B-cell antigens by using anti-B cell antisera trays kindly provided by Dr. Paul Terasaki, University of California at Los Angeles, utilizing techniques similar to A and B locus typing.

Data Analysis. Data from separate experiments are expressed as mean cpm of three to four replicate cultures with the SEM. Net counts per minute (E-C) were calculated by subtracting cpm of responses to unconjugated stimulators (C) from cpm of cultures with TNP-conjugated stimulator cells (E). E-C errors were determined by the formula for propagation of error. Stimulation indices (E/C) were calculated by dividing (E) by (C) values as defined above. Two-tailed rank-sum tests were performed to determine significant differences between experimental groups.

TABLE I

*Primary Responses of Fresh PBMCs to Autologous TNP-Conjugated Cells**

High responders‡	Unconjugated§	TNP-Conjugated	E-C	E-C Range‖
I	798 ± 139	20,813 ± 1,781	20,015 ± 1,951	11,370 - 20,015
Q	2,116 ± 984	17,920 ± 1,768	15,804 ± 2,023	6, 746 - 19,079
A	377 ± 48	7,533 ± 822	7,156 ± 825	6,760 - 15,507
E	587 ± 139	7,306 ± 726	6,718 ± 735	5,842 - 6,718
Low responders				
G	1,310 ± 198	4,520 ± 644	3,210 ± 674	194 - 4,087
N	566 ± 151	2,213 ± 682	1,646 ± 698	1,104 - 1,646
L	493 ± 49	871 ± 319	378 ± 323	187 - 378
K	322 ± 79	997 ± 50	675 ± 105	−472 - 675

* 1.5 × 10^5 responder cells, cocultured with 1.5 × 10^5 unconjugated or TNP-conjugated inactivated autologous cells, were pulsed with $[^3H]$thymidine for 20 h 5-6 days after culture in microtiter wells.

‡ High and low responders were distinguished using 2-7 separate experiments for each individual. Each low responder was assayed with a high responder in at least two experiments. High responders in all experiments had E/C values > 5, whereas low responders in all experiments had E/C values < 5.

§ Data are taken from representative experiments with triplicate or quadruplicate determinations.

‖ E-C range of all experiments performed for each subject.

Results

Primary TNP-Conjugate Responses. In vitro coculture of PBMCs with autologous TNP-conjugated cells stimulated primary proliferative responses in 16 of 18 individuals tested. Patterns of relative responsiveness were consistent and clearly differentiated high and low responders (Table I). 5 of 18 subjects tested were classified as low responders in that they always had stimulation indices of less than 5. High responders had an average stimulation index of 11 ± 1 (E/C ± SEM) compared with 2.1 ± 0.3 for low responders. This difference was not due to a shift in response kinetics, nor was it due to a general decrease in response capacity of low responder subjects in that high and low responders exhibited comparable proliferative responses to phytohemagglutinin and delayed skin test reagents (D. L. Peavy and M. F. Seldin, unpublished observations).

Studies of the effect of stimulator cell inactivation on primary proliferative responses to TNP-conjugated cells revealed no difference in the proliferative responses of lymphocytes from high responder subjects to stimulators inactivated with 4,000 rads compared with 500 rads. In contrast, low responder lymphocytes, did not respond to high-dose irradiated stimulator cells, although marginal but significant responses were generated with cells from three out of five low responder subjects when stimulator cells were not inactivated or when they were inactivated with 500 rads or with mitomycin C.

Secondary Responses. Restimulation of primed responder cells 3 wk after initial sensitization generated a secondary proliferative response to TNP-conjugated autologous PBMCs. The kinetics of the primary and secondary responses were easily distinguishable (Fig. 1). Primary responses showed little if any

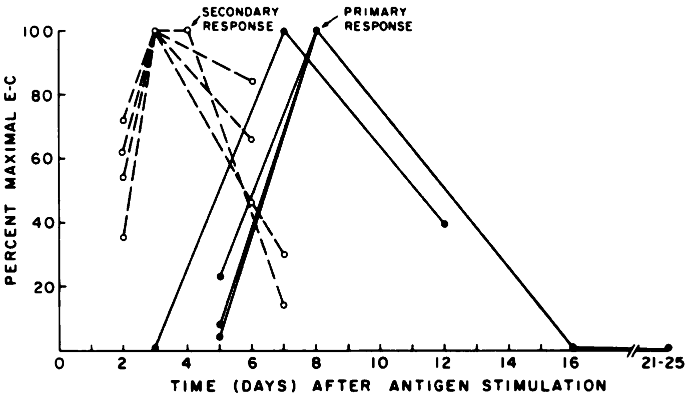

FIG. 1. Kinetics of primary and secondary proliferative responses to hapten-modified autologous cells. Data were taken from three separate experiments and normalized to percent maximal E-C response.

proliferation at day 3 and peaked at day 8; in contrast, secondary responses were maximal at day 3 and had diminished substantially by day 6. The peak secondary response was usually greater than the maximum primary response seen at a later time point. The stimulation index of secondary responses varied from 6.5–20 and averaged 14-fold at day 3. PBMCs precultured for 3 wk without stimulator cells generated responses consistent with primary responses, both in magnitude and kinetics, when TNP-conjugated stimulator cells were added after the preincubation period.

In general, cells from subjects which gave low primary responses failed to give responses after secondary restimulation. Low primary responder G (Table I), however, gave both primary and secondary responses when primary stimulators were mitomycin C-treated (Table II) or not inactivated (Table III), although no secondary response was seen when primary stimulators were treated with 4,000 rads. Secondary responses were not affected by inactivation of secondary stimulators with the high dose (4,000 rads) of γ-irradiation.

Preferential Restimulation with Autologous TNP-Conjugated Cells. To investigate whether secondary TNP-conjugate responses were dependent on recognition of some autologous determinants in addition to the hapten moiety, primed responders were restimulated with both autologous and heterologous TNP-modified PBMCs. The results of one such experiment are seen in Table II. In this experiment a self-preference in the secondary response was seen. That is, the response to autologous TNP-conjugated stimulators was much greater than that to allogeneic TNP-conjugated stimulators. In contrast to data reported in the mouse, in which cells primed with TNP-conjugated autologous cells were unresponsive to alloantigens (10), the response of autologous TNP-conjugate primed cells to alloantigen was often accelerated. Furthermore unprimed precultured cells developed proliferative responses following the usual kinetics of human MLC responses.

Influence of HLA-A and HLA-B Locus Determinants on Secondary TNP-Conjugate Responses. We next considered whether MHC determinants were involved in the recognition of TNP-conjugated cells. Fig. 2 shows a single experiment in which two responders were primed to autologous TNP-conjugated PBMCs and then restimulated with the same panel of three stimulators who

TABLE II

Self-Preference of Secondary Response to Hapten-Modified PBMCs

Stimulators* (HLA Phenotype) A	B		G Responders‡ Unprimed	Primed
G		Unconjugated	227 ± 34	756 ± 69
(1,2	27,40)	TNP-Conjugated	401 ± 71	10,998 ± 719
H		Unconjugated	1,945 ± 146	5,841 ± 146
(3,W33	15,17)	TNP-Conjugated	1,041 ± 164	5,877 ± 403

* TNP-conjugated or unconjugated stimulators were fresh PBMCs inactivated with 4,000 rads and cultured at 1.5 × 10^5/microtiter well. Cultures were harvested 68 h after secondary stimulation.

‡ Responders were cultured at 1.5 × 10^5 cells/microtiter well. Unprimed responders were fresh PBMCs. Primed responders were cocultured 3 wk with mitomycin C-inactivated trinitrophenylated autologous cells.

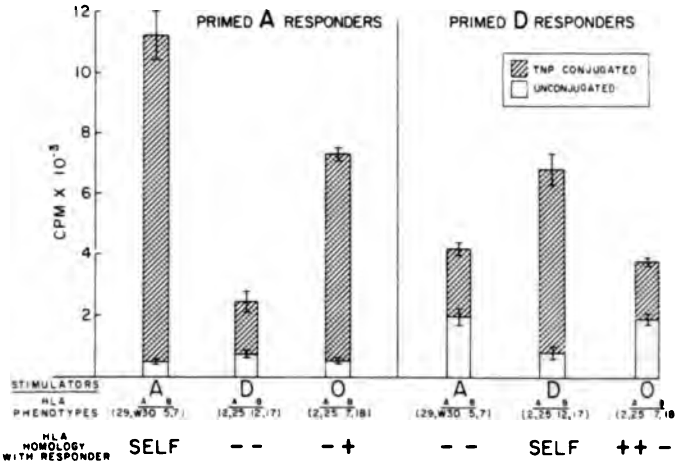

FIG. 2. HLA association of secondary responses. Primed responders were cocultured with 500 rad inactivated TNP-conjugated autologous cells and restimulated with 4,000 rad inactivated TNP-conjugated or unconjugated cells. Mean cpm ± SEM of responses to conjugated cells are represented by the total height of each bar, with the response to unconjugated cells shown in the clear area. As will be subsequently discussed (see text and Table IV) stimulator O shares a D region antigen (DRw2) with Responder A, in addition to the HLA-B7 antigen.

had been typed for HLA-A and HLA-B locus serologic specificities. For both responders there was a proliferative response to TNP-conjugated allogeneic cells in excess of that to alloantigens alone. The level of proliferation of one responder to TNP-conjugated cells that shared both HLA-A antigens was the same as the response to a completely HLA-nonidentical stimulator. That is, the responses of primed cells from subject D to stimulator cells O and A were essentially equivalent and were about 30% of that to autologous TNP-conjugated cells (E-C analysis). The response of the other responder (A) showed a much higher level of proliferation to allogeneic TNP-conjugated cells that shared an HLA-B antigen (O) than to completely HLA-nonidentical stimulator cells (D).

A further examination of the functional role of HLA-A and HLA-B antigen homologies in secondary TNP-conjugate responses was therefore performed (Table III). The presence or absence of HLA-A antigen homology did not affect

TABLE III

Effect of HLA A and B Locus Homology on Secondary Responses

TNP-Conjugated stimulators (HLA phenotype)			Primed G responder*			Primed M responder‡			Primed O responder		
			HLA Homology		E-C ± SEM§	HLA Homology		E-C ± SEM	HLA Homology		E-C ± SEM
	A	B	A-1,2	B-27,40		A-2,3	B-12,40		A-2,25	B-7,18	
GTNP$_x$	(1,2	27,40)	Self		15,588 ± 1,975			ND¶			ND
BTNP$_x$	(10,W32	5,W35)	–	–	361 ± 1,183			ND			ND
MTNP$_x$	(2,3	12,40)	+	+	750 ± 865	Self		19,436 ± 2,645	+	–	2,944 ± 255
OTNP$_x$	(2,25	7,18)			ND			ND	Self		6,074 ± 258
JTNP$_x$	(1,3	5,8)			ND			ND	–	–	2,105 ± 453
PTNP$_x$	(2,W31	5,12)			ND	+	+	4,634 ± 641	+	–	213 ± 401
TTNP$_x$	(W24,W31	7,14)			ND	–	–	9,181 ± 1,220	–	+	3,873 ± 530
CTNP$_x$	(1,9	7,8)			ND			ND	–	+	6,008 ± 372

* G primed responders were from cultures with autologous TNP-conjugated cells that were not inactivated.

‡ M primed and O primed responders were from cultures with autologous 4,000 rad inactivated TNP stimulators.

§ Secondary cultures of all responders were harvested 68 h after culture initiation.

TABLE IV
DRw Typing of Subjects

Subjects	B-Cell antigens*
G	No antigens detected
H	DRw1 (weak reaction)
B	Not typed
M	DRw2, DRw1, DRw6
O	DRw2
J	DRw3
P	DRw7
T	DRw7
C	DRw2, DRw3 (weak reaction)
A	DRw2
D	DRw7, DRw3 (weak reaction)

* 1977 WHO nomenclature for D-region related (DR) antigen (17).

the magnitude of responses, whereas HLA-B antigen homology correlated in some but not all, combinations with higher responses. One stimulator (C) which shared an HLA-B antigen with the primed responder (O) gave a proliferative response equivalent to autologous conjugated cells.

Influence of HLA-D Region Determinants on Secondary TNP-Conjugated Respones. In each experiment in which HLA-B antigen homology between stimulator and responder cells resulted in augmented responses the antigen shared was HLA-B7. Our attention was therefore directed to the *HLA-D* region, since *HLA-B7* is in strong linkage disequilibrium with *Dw2* (16).

Two alternative methods, MLC responses to homozygous typing cells (Dw specificities) and serologic detection of B-cell antigens can be used to determine D region alloantigens. The B-cell antigens utilized in our investigation have been designated DRw antigens, corresponding to the Dw specificities detected by the functional tests (WHO Conference 1977 [17]).

In the two examples where HLA-B antigen homology was clearly associated with a higher response than other allogeneic stimulators (Fig. 2, Responder A with Stimulator O: Table III, Responder O with Stimulator C) the individuals also shared the DRw2 antigen (Table IV). In addition the DRw2 antigen was also shared (Table III, Responder O) with a stimulator (M) that was not as effective as a DRw2 negative subject (T) in restimulating the response. It may be relevant in this regard that subject M was Black, whereas with the other DRw homologies the subjects were Caucasian.

More data relevant to the question of HLA-D region antigen association of the secondary proliferative response are shown in Table V. Stimulators that shared HLA-D region antigens with the responder cells generally gave higher responses, expressed as a percentage of the response with autologous stimulators (76.5 ± 5.3%; mean ± SEM), than did those that did not share these antigens (42.0 ± 7.0%). It should be noted that in experiment 1, stimulator Z was the son of responder A. Thus haploidentity in the one experiment where this was examined was not more effective than HLA-D antigen homology between allogeneic individuals. The data presented in this table also illustrate the finding that the response generated by allogeneic TNP-conjugated cells was

TABLE V

HLA-D Antigen Association of Secondary Proliferative Responses

Stimulator HLA phenotype A B	Primed A Responders					Primed O Responders				
	HLA Homology A	HLA Homology B	HLA-D Antigen* homology	% Maximal‡ E-C ± SEM		HLA Homology A	HLA Homology B	HLA-D Antigen homology	% Maximal E-C ± SEM	
				Exp. 1§	Exp. 2				Exp. 1	Exp. 3‖
ATNP$_x$ (29,W30 5,7)	Self			100 ± 1	100 ± 8	–	+	+	83 ± 2	
OTNP$_x$ (2,25 7,18)	–	+	+	88 ± 2	64 ± 1	Self			100 ± 1	100 ± 4
CTNP$_x$ (1,9 7,8)	–	+	+	69 ± 3		–	+	+	71 ± 3	99 ± 6
ZTNP$_x$¶ (1,W30 5)	+	+	+	60 ± 1		–	–		53 ± 2	
TTNP$_x$ (W24,W3 7,14)	–	+	–	39 ± 3		–	+	–	57 ± 4	64 ± 9
DTNP$_x$ (2,25 12, 17)	–	–	–	31 ± 1	17 ± 3	++	–	–	44 ± 1	

* Except for stimulator Z (see footnote ¶) the HLA-D antigen homology between responder and stimulator where indicated was in each case the DRw2 antigen (see Table IV), reflecting the strong linkage disequilibrium between HLA-B7 and this D region allele.

‡ Percent maximal E-C was calculated by determining the mean response to stimulators from triplicate or quadruplicate cultures and employing the following formula:

$$\frac{(\text{cpm allogeneic TNP-conjugated}) - (\text{cpm allogeneic unconjugated})}{[(\text{cpm syngeneic TNP-conjugated}) - (\text{cpm syngeneic unconjugated})]} \times 100\%.$$

§ In experiments 1 + 2 primed responders (A and O) were generated by coculture with 500 rad inactivated autologous trinitrophenylated cells. Secondary cultures were harvested at 48 h.

‖ In experiment 3 primed O responders were generated by coculture with 4,000 rad inactivated autologous trinitrophenylated cells. Secondary responses were harvested at 68 h.

¶ Stimulator Z is the son of Responder A.

not entirely explained on the basis of shared MHC specificities, insofar as they were detected.

HLA-D region determinant homology between primed responder and stimulator correlated significantly with the magnitude of the secondary TNP-conjugate proliferative response. The hypothesis that the two samples (those sharing and not sharing HLA-D antigens) were drawn from the same population was rejected ($P < 0.01$; two-tailed rank-sum test). On the other hand, stimulators with HLA-A or HLA-B homology with the responder (excluding stimulators where HLA-D antigen homology with the responder was also present) were not significantly different from stimulators with no responder homology ($P > 0.2$).

Discussion

This investigation was undertaken to develop an in vitro model for examination of the functional significance of MHC determinants in human immune responses. We studied proliferative responses of human lymphoid cells which had been conjugated to a well-defined hapten (TNP), based on the previous findings of MHC association in murine responses (10). Recently methods for obtaining primed proliferative and cytotoxic responses to hapten-modified human lymphoid cells have been reported (11, 18). Although self-preference was suggested, the role of the MHC, if any, in these responses was not established. Our experiments demonstrate that under appropriate conditions human lymphocytes develop both primary and secondary responses in vitro to TNP-conjugated lymphoid cells. Primary proliferative responses have not been previously reported for either human or murine TNP-conjugate systems.

Based on primary proliferative responses to TNP-conjugated autologous cells, individuals could be segregated into high and low responders. Five of 18 subjects studied were classified as low responders. Three of these low responders generated minimal primary responses but were extremely sensitive to stimulator cell inactivation. A likely explanation for this observation is that functional stimulator cells are required in greater numbers or for a longer time period to trigger responses in these individuals. This in turn may be caused by decreased efficiency in antigen presentation, or recognition, or both.

Although we can only speculate on mechanisms responsible for high and low responsiveness, it is unlikely that previous exposure (priming) to TNP had occurred. Furthermore these same subjects did not segregate into high and low responders when stimulated with mitogens or delayed skin test reagents. Similar phenomena in proliferative responses to soluble antigens (4) and cytotoxic responses to TNP-conjugated cells (6) in the mouse have been explained on the basis of *Ir* genes encoded within the *I* region of *H-2*, which is analogous to the *HLA-D* region. The possibility that high and low responsiveness to TNP-conjugates in humans may reflect presentation or recognition of particular *D* region gene products, rather than responsiveness to TNP per se, is therefore particularly interesting. Responsiveness to other haptens in the same system may resolve this possibility.

A major interest has been the possible involvement of MHC gene products in stimulation of secondary responses. The data clearly demonstrate self-preference in the secondary stimulation phase, although various levels of responsiveness to allogeneic TNP-conjugated cells were seen. Several possible mechanisms were considered to explain both self-preference and apparent reactivity to TNP on allogeneic cells in the secondary response. (*a*) Since alloantigens can stimulate suppressor T-cell function (19, 20) the apparent self-preference of the secondary response may be due to the absence of alloantigen determinants rather than a requirement for autologous structures. We consider this explanation unlikely because of the accelerated kinetics of the secondary response. Furthermore, preliminary experiments demonstrate that the addition of allogeneic cells to secondary cultures does not suppress responses to autologous TNP-conjugated stimulators. (*b*) Part or all of the response may be to TNP-protein on the cell surface without special regard to HLA. Self-preference may, therefore, be due to autologous proteins having unique determinants. While not formally excluded, this possibility conflicts with the findings in murine models and would not explain our findings of preferential restimulation with partial HLA-D region homologous stimulators. (*c*) There may be a requirement comparable to that in murine systems for certain MHC determinant homologies. Our data did strongly suggest that D region homology between primed responder and stimulator account for increased responsiveness. It should be stated, however, that the limited number of D region alleles which can be currently detected constrain this conclusion. It is possible, of course, that the requisite genetic homologies between responder and stimulator cells are not with detected alloantigenic determinants, but with other HLA-D region gene products in linkage disequilibrium with the typed alleles. In murine secondary proliferative responses to TNP-conjugated cells a strict MHC homology restriction was seen, but intra-*H-2* mapping was complicated (10). *K* + *I* region homology was more effective in restimulating secondary responses than *I* region homology alone. Since all subjects in this study with DRw2 homology also shared the HLA-B7 antigen a similar cooperative interaction between HLA-B and D region determinants must be considered.

It must be emphasized that allogeneic TNP-conjugated cells which did not share any detected MHC determinants with the primed responder still frequently stimulated a measurable proliferative response, albeit substantially less than autologous cells. The role of the human MHC in T-cell-mediated responses to conventional antigens is not yet clear. Differences between apparent mouse and human homology requirements may be explained by more extensive crossreactivities between human than murine MHC determinants (i.e., differences between outbred and inbred populations). Recent work with wild mice has shown that cross-reactivities in the TNP cell-mediated lympholysis assay do occur in *H-2* disparate hosts when the *H-2* haplotypes between stimulator and target cells are closely related (21). A less likely explanation is the possibility that the recognitive structures in human lymphocytes are less discriminating than murine receptors. That is, greater cross-reactivity at the receptor level rather than the stimulator determinant level could account for disparities.

Alternatively, the apparently nonrestricted component of the response may represent proliferation induced by TNP-conjugated allogeneic proteins presented by syngeneic macrophages. Since syngeneic macrophages are present in both the primary and secondary phase, MHC restriction requirements would not preclude a proliferative response dependent on macrophage handling of TNP-conjugated proteins on the cell surface. The much lower percentage of macrophage-monocytes in murine spleen cell populations (3-5%) than in human PBMC populations (20-25%) could explain the strict homology requirements in secondary TNP-conjugate proliferative responses reported in the mouse (10).

Recently Dickmeiss et al. (22) reported that cell-mediated cytotoxicity against hapten-conjugated lymphocytes is HLA restricted. They utilized dinitrophenylated PBMCs from subjects who had been contact-sensitized in vivo with dinitrochlorobenzene or dinitrofluorobenzene. After an additional in vitro sensitization phase, the cytotoxic responses were largely restricted to *HLA-A* or *HLA-B* locus homologous hapten-conjugated targets. Thus, their findings with cytotoxicity contrast with our results that human proliferative responses to hapten-conjugated cells are associated with the HLA-D region, and both sets of data are in accord with previous studies in mice (6, 10).

The role of HLA determinants in recognition and/or presentation of conventional antigens may help elucidate the pathogenesis of a variety of diseases. The association of a number of human diseases with certain *HLA* alleles, probably a reflection of linkage disequilibrium, has become apparent in recent years (1, 2). Moreover, juvenile diabetes mellitus (23, 24) and ragweed hayfever (25), which are not associated with particular *HLA* alleles but segregate with the MHC in family studies, may be caused in part by expression of *Ir* genes encoded in the *D* region. We found that secondary proliferative responses of human lymphocytes to TNP-conjugated lymphoid cells are associated with the HLA-D region. Thus the model we describe has important implications for definition of human T cell recognitive units and may permit a functional characterization of the role of HLA determinants in both normal and pathologic states.

Summary

An in vitro model was developed to study both primary and secondary proliferative responses of human lymphocytes to hapten-conjugated peripheral blood mononuclear cells. Coculture of human lymphocytes with autologous trinitrophenyl (TNP)-conjugated stimulator cells resulted in primary proliferative responses. Subjects segregated into high and low primary responders with mean stimulation indices of 11 and 2.1, respectively. Restimulation of primed cells from high responder subjects 3 wk after initial sensitization generated secondary proliferative responses.

To investigate the antigenic requirements for secondary stimulation, autologous TNP-conjugate primed responders were restimulated with both autologous and allogeneic TNP-conjugated stimulators. In all experiments restimulation with autologous conjugated cells yielded substantially greater proliferative responses than with allogeneic conjugates. Experiments were then performed to ascertain whether HLA determinant homology between primed responder and stimulator cells influenced the level of secondary responsiveness. Homology for HLA-A and B locus serologic determinants was not associated with enhanced responsiveness. In contrast, D region determinant homology, detected by B-cell

antigen typing, showed a highly significant positive correlation with the magnitude of secondary responses. The data thus strongly suggest that for secondary proliferative responses to TNP, human T cells recognize hapten in association with HLA-D region determinants.

We wish to thank Shirley Qualia and Kathy Freeman for excellent technical assistance in HLA serologic and B-cell antigen typing and Dr. Paul I. Terasaki and Mr. Ken Slyker for making B-cell typing trays available. The advice of Dr. Wes Bullock and Dr. Joe Templeton and the assistance of Dan Kastner in data analysis are gratefully acknowledged. We are indebted to Mrs. Elaine Bowers for her perseverance in the preparation of this manuscript.

Received for publication 17 January 1978.

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INTERACTION OF GLUCOCORTICOIDS WITH MACROPHAGES

Identification of Glucocorticoid Receptors in Monocytes and Macrophages*

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The mononuclear phagocyte system plays a central role in mediating host responses in inflammation (1). Glucocorticoids have anti-inflammatory actions that may be of considerable importance in the therapeutic effects of these agents in chronic inflammation; it is possible that some of these effects are mediated through direct hormonal action on macrophages. Although the site of action of the glucocorticoids on macrophages has not been established, it has been shown that in many other glucocorticoid target systems the effects of glucocorticoids are mediated by specific macromolecular binding proteins, referred to as receptors (2-4).

In this study we have established that monocytes and macrophages contain saturable glucocorticoid-binding proteins, with specificity of binding for cortisol, corticosterone, and related synthetic steroids such as dexamethasone, and that they have dissociation constants for binding within physiological ranges.

Materials and Methods

Cell Culture. Mouse peritoneal macrophages were harvested from female Swiss Webster mice (CD-1 and CF-1; Charles River Breeding Laboratories, Inc., Wilmington, Mass.) weighing 20-25 g, as described previously (5, 6). Resident macrophages were obtained from unstimulated animals, and elicited macrophages were obtained from mice injected 4 days before with 1.0 ml of Brewer thioglycollate medium (5, 6). Cells were plated at 0.5-1 × 10^6 cells/well in 2-cm^2-diameter multiwell plates (Microbiological Associates, Walkersville, Md.) in Dulbecco's modified Eagle's medium high glucose formulation (DME)[1] supplemented with 10% heat-inactivated fetal calf serum (FCS) (Grand Island Biological Co., Grand Island, N. Y.). 1-24 h before binding experiments, cells were placed in serum-free DME supplemented with 0.2% lactalbumin hydrolysate DME-LH).

Rabbit alveolar macrophages were obtained by lung lavage 10-14 days after intravenous

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* Supported by grants AM 14780, AM 03535, and CA 17323 from the U. S. Public Health Service, by grants from the National Chapter of the Arthritis Foundation and the Dufault Bequest for Arthritis Research administered by the New Hampshire Chapter of the Arthritis Foundation, and by the U. S. Department of Energy.

[1] *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; DME-LH, DME supplemented with 0.2% lactalbumin hydrolysate; FCS, fetal calf serum; K_d, half-maximum saturation; KRPg, Krebs-Ringer phosphate buffer containing 5.5 mM glucose.

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injection of 0.15 ml of Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.) (7). The cells were washed 3 times in Hanks' balanced salt solution and resuspended at 2-4 × 10^6/ml in DME-LH. The alveolar macrophages were then plated in multiwell dishes at 2 × 10^6 cells/ well, or cultured in suspension for 3 h before use in the assays of steroid binding. Cells in suspension were centrifuged, washed in Hanks' balanced salt solution, and resuspended at cytocrits of 0.24–0.36 for binding experiments.

Human monocytes were isolated from 300 ml of freshly drawn, citrated blood. Mononuclear cells were concentrated by Ficoll-Hypaque fractionation (8), washed twice in RPMI-1640 medium (Grand Island Biological Co.), and resuspended at 1 × 10^7 cells/ml in RPMI-1640 supplemented with 10% autologous serum. Cells were plated in multiwell plates precoated with 10 μg of fibrin/ cm^2 to aid adherence of monocytes. Nonradioactive fibrin layers were prepared by the procedure used for ^{125}I-labeled fibrin layers (9). After 16 h at 37°C in a CO_2 incubator, nonadherent cells were removed by washing, and adherent cells were placed in serum-free DME-LH for 2 h before measurement of receptors. The mean purity of the monocyte preparation was 93%.

Cells were counted in a Coulter counter (Coulter Electronics Inc., Hialeah, Fla.), and differential cell counts were made on cytocentrifuge preparations stained with Wright's stain.

Steroid Binding

MEASUREMENT OF STEROID BOUND TO RECEPTORS IN INTACT CELLS. Monolayers of macrophages in 2-cm^2-diameter wells were washed rapidly 3 times at room temperature with ≅2 ml of Krebs-Ringer phosphate buffer containing 5.5 mM glucose (KRPg). Each well then received 100–200 μl of KRPg containing [1,2,4-^{3}H (*N*)]dexamethasone or [6,7-^{3}H(*N*)]dexamethasone (New England Nuclear, Boston, Mass.; 30-50 Ci/mmol). In a few experiments, [1,2,3-^{3}H(n)]triamcinolone acetonide (New England Nuclear; 45 Ci/mmol) was used instead of [^{3}H]dexamethasone. The plates were incubated at 37°C for 70 min, then placed on ice. Aliquots of medium from each well were taken to determine the concentration of free [^{3}H]dexamethasone. Each well was rinsed 6 times with 2 ml of ice-cold KRPg to remove free steroid, and then left for 20 min at room temperature with buffer. The treatment at room temperature removed most of the nonsaturable fraction of cell-associated steroid, as previously shown for thymus cells (10, 11). The remaining steroid was extracted with 150 μl of ethanol at room temperature for 20 min, followed by an additional 50 μl to rinse the wells. Samples were counted in Bray's fluid (New England Nuclear) in a Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Data were analyzed by conventional plots of bound versus free steroid, and by Scatchard plots separated by trial and error into saturable and nonsaturable components (12, 13).

Protein content of monolayers was determined in sham-treated wells which had not been extracted with ethanol, and on residual cellular protein after ethanol extraction by solubilization in 0.5 M NaOH followed by Lowry-Folin procedure with crystalline bovine serum albumin as standard (14).

COMPETITIVE BINDING STUDIES. Competition for binding with whole cells was carried out as for binding of [^{3}H]dexamethasone alone, except that the incubation media contained both [^{3}H]dexamethasone and the unlabeled competing steroid at the concentrations indicated in Results. Cortisol and other steroids of similar solubility were dissolved in KRPg, and their concentration was determined spectrophotometrically by measuring adsorption at 250 nm and assuming $\epsilon = 1.4 \times 10^4\ M^{-1}\ cm^{-1}$ (11). Less soluble steroids such as estradiol were dissolved in ethanol; incubations for these steroids contained up to 1% ethanol, which did not affect dexamethasone binding. Steroids were obtained from Steraloids, Inc., Pawling, N. Y., Calbiochem, San Diego, Calif., or Sigma Chemical Co., St. Louis, Mo.

DEXAMETHASONE BINDING TO "CYTOPLASMIC" AND NUCLEAR FORMS OF GLUCOCORTICOID RECEPTORS AND TEMPERATURE-SENSITIVE TRANSLOCATION FROM "CYTOPLASMIC" TO NUCLEAR FORMS. Thioglycollate-elicited macrophages were either washed out of the peritoneal cavity of mice and used directly, or cultured for 24 h in 100-mm-diameter tissue culture dishes as described above, and scraped off the surface with a rubber policeman before use. The cells were sedimented and washed three times with KRPg at room temperature, and finally incubated at a cytocrit of 0.33 ml packed cells/ml of suspension with [^{3}H]dexamethasone in the absence or presence of 500 nM unlabeled dexamethasone. The values obtained in the presence of unlabeled dexamethasone were for nonsaturable binding. The cells were incubated at 3°C for 120 min, then at 37°C for 30 min. After both incubations, the cells in duplicate 20-μl aliquots of the suspensions were disrupted by

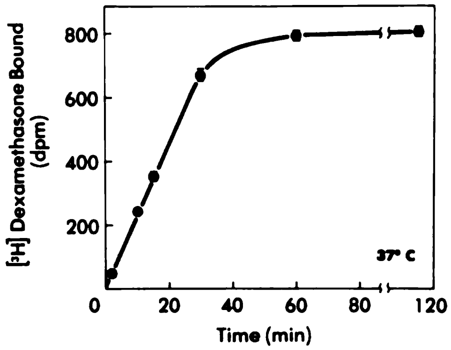

FIG. 1. Time course of binding of dexamethasone to glucocorticoid receptors in thioglycollate-elicited mouse peritoneal macrophages. Binding to cells was with 2.8 nM [^{3}H]dexamethasone. Only saturable binding is shown. The nonsaturable component determined in the presence of added cold dexamethasone (1,000 nM) was <20% of the total binding. Results are expressed as mean ± SD, n = 3.

hypotonic shock in 1.5 mM $MgCl_2$ to determine "cytoplasmic" and nuclear binding by a modification of the charcoal absorption and nuclear pellet procedures developed for lymphocytes (10, 15). A modification consisting of a freeze-thaw (freezing in acetone-dry ice, thawing at 3°C) after addition of the cells to $MgCl_2$ was necessary to break open the macrophages. Values were expressed as the mean ± SD for saturable binding, obtained by subtracting the mean for nonsaturable binding at 4 nM [^{3}H]dexamethasone.

Results

Physicochemical Interactions of Dexamethasone with Thioglycollate-Elicited Mouse Peritoneal Macrophages. Specific binding of [^{3}H]dexamethasone to monolayers of thioglycollate-elicited mouse peritoneal macrophages increased rapidly for 30 min at 37°C, and then reached a plateau (Fig. 1). The rate of binding at 3°C was slower (not shown). Binding could be resolved into two components: a high affinity component, which was saturable and could be displaced by excess nonradioactive steroid, and a low affinity component, which was not saturated up to 100 nM dexamethasone and was not displaced by excess nonradioactive steroid (Fig. 2). The high affinity binding had a half-maximum saturation (K_d) at 3.7 nM dexamethasone. High affinity binding was not influenced by the prolonged washing procedures used, whereas low affinity (nonsaturable) binding usually decreased to <20% of the total binding of radioactive hormone; the 20-min room temperature wash was particularly useful in decreasing nonsaturable binding. High affinity binding was ≅ 80–100 fmol of dexamethasone/mg cell protein (3,500–6,000 binding sites/cell) (Table I). Binding was enhanced by ≅20% when the macrophages were removed from DME + FCS and placed in DME-LH at least 1 h before experiments for hormone binding. High affinity binding did not increase further with culture of the cells in DME-LH for up to 72 h. The number and avidity of the hormone receptors did not change with time. High affinity binding was the same when macrophages that had been explanted 1, 24, 72, or 144 h were used. Binding was proportional to the number of macrophages adhering to the plate. Only viable cells bound the steroid; macrophages killed by heat or acid did not bind dexamethasone.

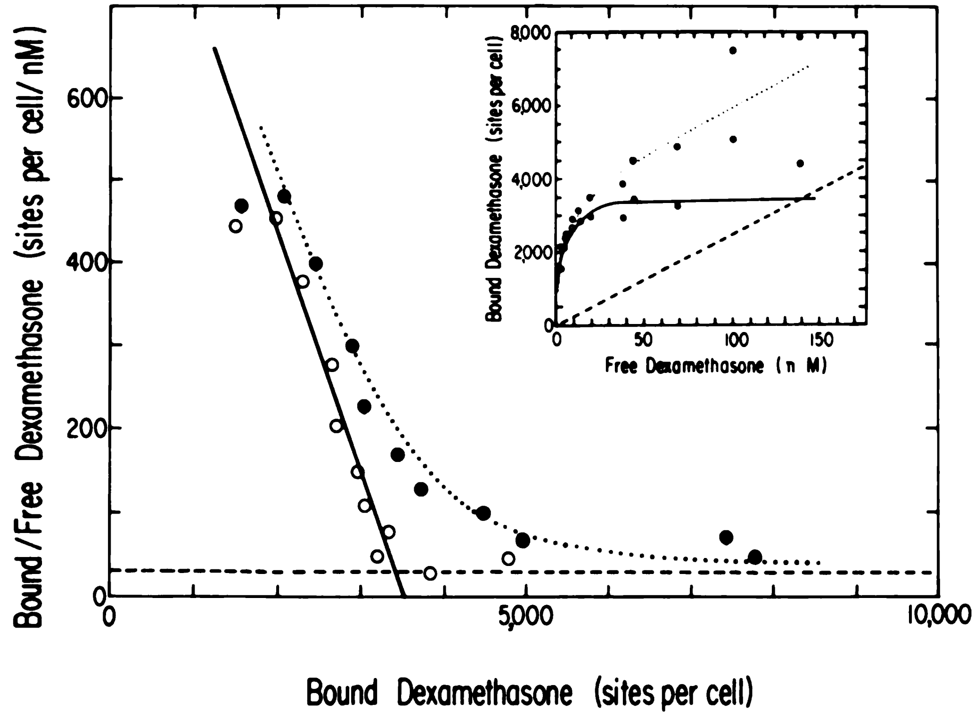

FIG. 2. [^{3}H]dexamethasone binding of thioglycollate-elicited macrophages. The binding of dexamethasone to intact cells in monolayer at 37°C was made as described in Materials and Methods. The results of a typical experiment are shown as a Scatchard plot analyzed into saturable (——) and nonsaturable (- - -) components, and as a conventional plot of bound vs. free dexamethasone (inset). The parameters of the solid lines are: slope = $-K_a$ = 0.27 (nM)$^{-1}$, intercept = 3,500 sites/cell. The line (···) gives the sum of the two components. Original data are shown by (●) and original data after subtraction of the nonsaturable component are shown by (○).

TABLE I

Characteristics of Glucocorticoid Receptors in Cultured Mononuclear Phagocytes

Source	Species	Receptors			
		[^{3}H]Labeled ligand	fmol/mg of cell protein	Sites/cell	K_d
					nM
Blood monocytes	Human	Dexamethasone	470	9,000	7.7
Resident peritoneal macrophages	Mouse	Triamcinolone acetonide	270	6,100	2.1
Exudative peritoneal macrophages	Mouse	Dexamethasone	80	4,300	3.7
P388D1 cells	Mouse	Dexamethasone	65	4,200	4.0
Induced alveolar macrophages	Rabbit	Triamcinolone acetonide	3,800*	4,500	2.6
		Dexamethasone	85	4,600	1.8

* fmol/ml packed cells.

TABLE II

Competition by Various Steroids for Binding of [^{3}H]Dexamethasone to Glucocorticoid Receptors in Plated Thioglycollate-Elicited Mouse Macrophages

Competing steroid	Concentration	[^{3}H]Dexamethasone binding*
	nM	*% control*
Dexamethasone	1,000	18 ± 1
Cortisol	100	77 ± 1
	1,000	33 ± 1
Corticosterone	100	84 ± 6
	1,000	40 ± 2
Cortexolone	90	92 ± 3
	900	56 ± 2
Progesterone	120	93 ± 3
	1,200	54 ± 3
Cortisone	1,000	81 ± 2
11-Epicortisol	1,000	105
Estradiol	100	98 ± 8
	1,000	96 ± 10
Dihydrotestosterone	100	101 ± 15
	1,000	85 ± 11
Testosterone	85	85 ± 10
	850	78 ± 3

* Whole cell binding assay; values are shown as mean ± range. [^{3}H]Dexamethasone was present in the assay at 8 nM.

The binding of dexamethasone to the thioglycollate-elicited macrophages was specific for glucocorticoids. As shown in Table II, dexamethasone, cortisol, and corticosterone competed for receptor-bound [^{3}H]dexamethasone. Progesterone and cortexolone also competed for receptors. These substances sometimes act as antiglucocorticoids (4). Cortisone had a small but reproducible competing effect in macrophages, and it is possible that some limited metabolic conversion to cortisol may have occurred. Estradiol, 11-epicortisol, testosterone, and dihydrotestosterone did not compete for binding.

In many other glucocorticoid-responsive systems, the hormone effect depends upon glucocorticoid binding to a receptor protein in the "cytoplasm" followed by intracellular transport into the nucleus (4). In a modified cytosol and nuclear-pellet preparation at 3°C, the receptor-bound dexamethasone in thioglycollate-elicited macrophages was largely found in a soluble "cytoplasmic" form, which was rapidly transferred to a nuclear form when the temperature was raised to 37°C (Fig. 3). Thus, these results support the presence of a functional receptor system for dexamethasone in elicited mouse macrophages.

Glucocorticoid Receptors in Resident Mouse Peritoneal Macrophages. Macrophages normally residing in the peritoneal cavity of mice also bound glucocorticoids with high affinity (for triamcinolone acetonide, K_d = 2.1 nM) and contained ≅6,000 sites/cell (Table I). As in elicited peritoneal macrophages, steroids with glucocorticoid activity (triamcinolone acetonide and dexamethasone) and progesterone competed with dexamethasone for binding to receptors (Table III).

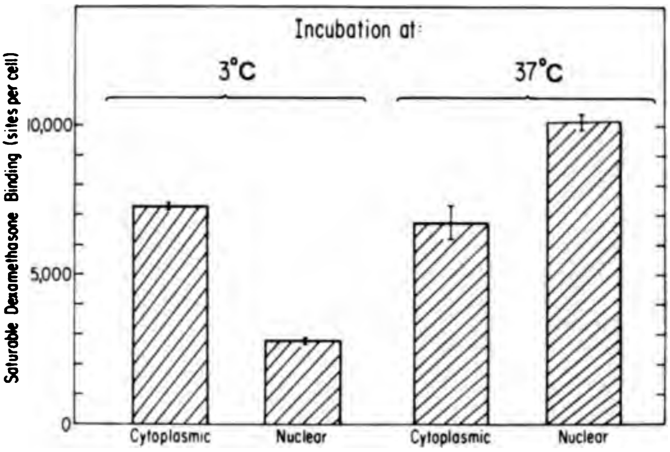

FIG. 3. Dexamethasone binding to "cytoplasmic" and nuclear forms of glucocorticoid receptors in thioglycollate-elicited mouse peritoneal macrophages and temperature-sensitive translocation from "cytoplasmic" to nuclear forms. Cell suspensions were incubated with [^{3}H]dexamethasone as described in Materials and Methods. Saturable binding sites in "cytoplasmic" fractions and nuclear pellets for cells incubated at 3°C for 120 min are shown on the left; sites for cells incubated at 3°C for 120 min followed by 37°C for 30 min are shown on the right. Values are expressed as mean and range obtained for binding at 4 nM [^{3}H]dexamethasone.

Mouse Macrophages from Other Sources. High affinity saturable binding of glucocorticoids to macrophages elicited in the peritoneal cavity by pristane (data not shown), resident lung macrophages (data not shown), and P388D1 cells from a macrophage-like continuous line[2] (Table I) was also demonstrated.

Rabbit Alveolar Macrophages. Pulmonary alveolar macrophages obtained from rabbits 2 wk after intravenous injection of complete Freund's adjuvant bound dexamethasone and triamcinolone acetonide to high affinity receptor sites (Table I). Cells adhering to plastic surfaces and cells in suspension bound the glucocorticoid equally well. The number and affinity of the rabbit macrophage glucocorticoid receptors were similar to that observed for mouse macrophages. For optimal binding, the macrophages were cultured in serum-free DME-LH for 2-24 h after removal from the lungs. Dexamethasone bound specifically to receptors with high affinity for glucocorticoids. As shown in Fig. 4, dexamethasone, triamcinolone acetonide, and hydrocortisone competed with [^{3}H]dexamethasone for binding to receptors, whereas cortisone, estradiol, and dihydrotestosterone did not.

Human Blood Monocytes. The rabbit and the mouse are glucocorticoid-sensitive species, whereas man is relatively glucocorticoid resistant (2). It was thus of particular interest to examine the glucocorticoid receptors in human mononuclear phagocytes. Blood monocytes in monolayer culture bound dexamethasone (Fig. 5). The high affinity component was saturated by 30 min of incubation at 37°C with 10 nM dexamethasone, and it had a K_d of $\cong$7 nM. The low affinity component was nonsaturable. The human monocytes had $\cong$9,000

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[2] Werb, Z., R. Foley, and A. Munck. 1978. Glucocorticoid receptors and glucocorticoid-sensitive secretion of neutral proteinases in a macrophage line. *J. Immunol.* In press.

TABLE III

Competition by Various Steroids for Binding of [^{3}H]Dexamethasone to Glucocorticoid Receptors in Resident Mouse Peritoneal Macrophages

Competing steroid	Concentration	[^{3}H]Dexamethasone binding*
	nM	*% control*
None	–	100 ± 3
Triamcinolone acetonide	10	34 ± 0
	100	29 ± 1
Dexamethasone	100	32 ± 1
	1,000	18 ± 1
Cortisol	100	63 ± 12
	1,000	30 ± 8
Cortisone	1,000	82 ± 6
Progesterone	100	73± 6
	1,000	38 ± 9
11-Epicortisol	1,000	107 ± 8
Dihydrotestosterone	1,000	100 ± 2
Estradiol	1,000	93 ± 4

* Whole cell binding assay with [^{3}H]dexamethasone present in the assay at 3 nM; values are shown as mean ± SD of triplicates.

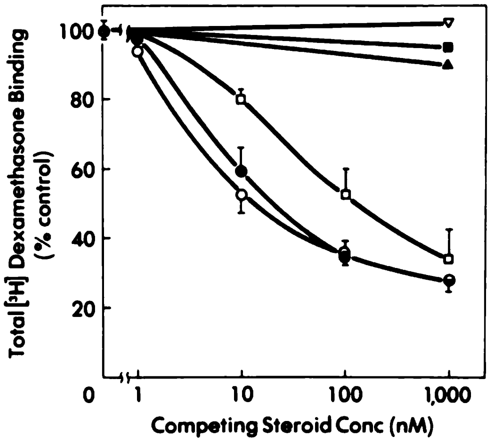

FIG. 4. Competition by various steroids for binding to glucocorticoid receptors in adherent rabbit alveolar macrophages. Competition for binding of [^{3}H]dexamethasone at 10 nM was tested with dexamethasone (●), triamcinolone acetonide (○), cortisol (□), cortisone (▲), estradiol (▽), and dihydrotestosterone (▼). Results are expressed as mean ± SD, n = 3. Conc, concentration.

sites/cell (Table I), well within the range for rabbits and mice. There were reproducibly more glucocorticoid receptors in blood monocytes than in the lymphocytes isolated from the same preparations (≅3,000 sites/cell), and platelets contaminating the monocyte preparations contained no demonstrable receptors (G. Crabtree, K. Smith, and A. Munck, unpublished observations). Cortisol and dexamethasone competed for binding of [^{3}H]dexamethasone to the monocytes, whereas steroids without glucocorticoid activity did not (Table IV).

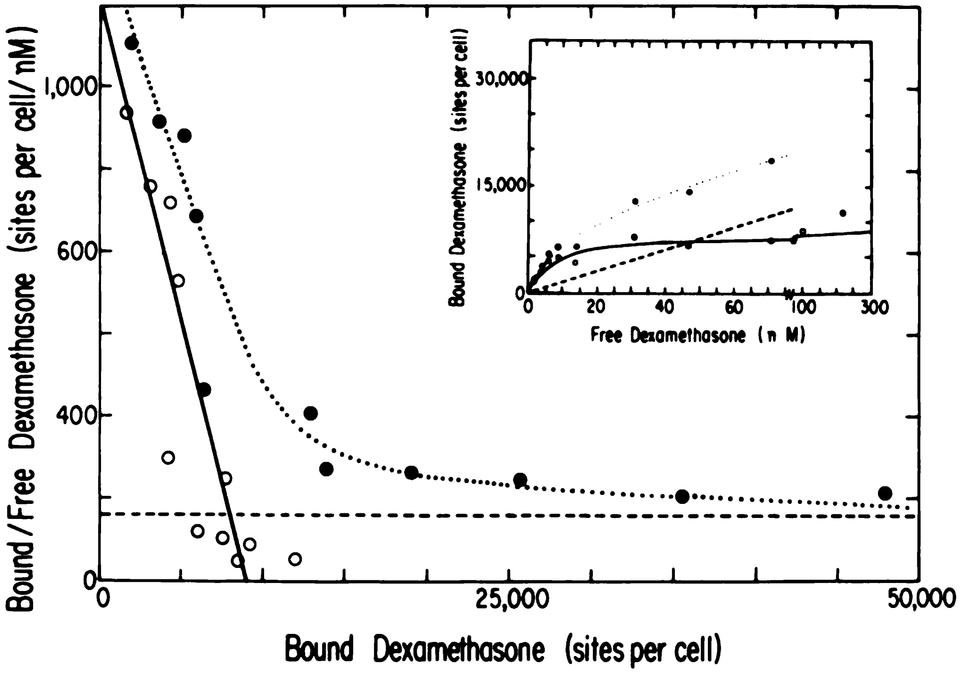

FIG. 5. [^{3}H]dexamethasone binding to monolayers of human monocytes. Scatchard and conventional (inset) plots of bound vs. free dexamethasone are used to determine saturable and nonsaturable components. For details, see Fig. 2. The solid line parameters are: slope $= -K_a = 0.13\ (\text{nM})^{-1}$, intercept = 9,000 sites/cell.

TABLE IV

Competition by Various Steroids for Binding of [^{3}H]Dexamethasone to Glucocorticoid Receptors in Human Monocytes

Competing steroid	Concentration	[^{3}H]Dexamethasone binding*
	nM	*% control*
Dexamethasone	1,000	22 ± 3
Cortisol	1,000	59 ± 3
Cortisone	1,000	95 ± 3
11-Epicortisol	1,000	118 ± 9

* Whole cell binding assay; values are shown as mean ± SD. [^{3}H]Dexamethasone was present in the assay at 8 nM.

Thus, the monocyte contained specific binding sites consistent with glucocorticoid action at physiological concentrations.

Discussion

The studies reported here show that glucocorticoid receptors are present in intact mononuclear phagocytes from mouse, rabbit, and man. The dissociation constants found (Table I) were ≅2–8 nM for dexamethasone; the competition for binding by cortisol at about 10-fold higher concentrations suggests that glucocorticoid receptor-mediated functions in macrophages would be operative at physiological concentrations.

The number of receptor sites and their affinity were similar for resident macrophages, macrophages elicited by inflammatory stimuli, and blood monocytes, and they were similar to those reported for lymphoid cells (3, 4, 16). Although unstimulated monocytes and macrophages had higher concentrations of steroid receptors than did elicited macrophages, this does not necessarily signify a difference in sensitivity to glucocorticoid action. Mitogen-stimulated lymphocytes contain several times more receptors than unstimulated lymphocytes, and yet both populations are equally steroid sensitive (16).

In the present study, glucocorticoid binding was examined with dexamethasone and triamcinolone acetonide as the ligands. These synthetic glucocorticoids have the advantage of not binding to transcortin, a serum protein that binds cortisol (17); thus, accurate glucocorticoid concentrations could be determined. Also, experiments were made in the absence of serum to decrease interference from exogenous steroids. Serum cortisol and progesterone both compete with dexamethasone for binding, and could influence the free steroid concentrations present in the experiments.

In other work we have shown that P388D1 macrophages, a continuous cell line, contain glucocorticoid receptors indistinguishable from those of normal mouse macrophages.[2] We have demonstrated temperature-sensitive transfer of glucocorticoid receptors to macrophage nuclei. With P388D1 cells we have been able to demonstrate glucocorticoid binding directly to proteins isolated from cytoplasm, temperature-dependent activation of the hormone-receptor complex, and translocation of only activated complexes into isolated nuclei.[2]

In virtually every target tissue investigated, the first step in steroid hormone action is the binding of steroid to specific receptor proteins. Because their specificity and affinity correlate with biological functions, they may mediate the hormone action. Some effects of glucocorticoids on macrophages have been described previously; in most experiments, however, pharmacological concentrations of steroids were used (2, 18). These effects are unlikely to be mediated by the high affinity receptors described in this paper. In the accompanying paper (18) hormone effects at glucocorticoid concentrations similar in specificity and affinity to those for receptor characteristics are shown for macrophage secretion and growth.

Summary

Glucocorticoid binding was measured in resident and thioglycollate-elicited mouse peritoneal macrophages, rabbit alveolar macrophages, and human monocytes. Two assays of binding were used—an assay with intact cells in suspension or monolayers, and an assay of cytosol and nuclear forms of glucocorticoid receptors. The mononuclear phagocytes contained $\cong 4$–10×10^3 high affinity receptor sites per cell, with dissociation constants of $\cong 2$–8 nM dexamethasone. The binding to the saturable sites was specific for steroids with glucocorticoid or antiglucocorticoid activity. Cortisol, corticosterone, and progesterone competed with dexamethasone for binding, whereas estradiol, dihydrotestosterone, and 11-epicortisol competed very little.

Binding of dexamethasone to cytosol and nuclear forms of the receptor complex and temperature-sensitive translocation of cytosol forms to nuclear forms were shown. At 37°C the predominant form of the hormone-receptor complex was nuclear. These results demonstrate that corticosteroids interact with macrophages at physiological concentrations.

We thank Ms. I. Vander Heiden and Ms. Nancy Cidlowski for their technical assistance. These studies were initiated while Z. W. was a visiting professor in the laboratory of Dr. E. D. Harris, Jr., Dartmouth Medical School.

Received for publication 27 January 1978.

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BIOCHEMICAL ACTIONS OF GLUCOCORTICOIDS ON MACROPHAGES IN CULTURE

Specific Inhibition of Elastase, Collagenase, and Plasminogen Activator Secretion and Effects on Other Metabolic Functions*

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Glucocorticoids have a major role in the therapy of inflammatory and immunologically mediated diseases. The precise mechanism of the suppressive and anti-inflammatory effects of these drugs is still unknown, but the functions of the mononuclear phagocyte system are generally believed to be sensitive to glucocorticoid action (1-3). During glucocorticoid administration in vivo, monocytopenia occurs (4-6) and monocytes fail to accumulate at inflammatory sites (7-10). In the presence of glucocorticoids, macrophages do not respond to macrophage migration inhibitory factor (11), and fail to become "armed" or activated (8, 11-13). In experiments in vivo it is difficult to determine whether glucocorticoids are acting directly on macrophages or indirectly on lymphocytes and other cells that produce mediators of macrophage function (3, 8).

Macrophages may serve as a direct target for the therapeutic actions of anti-inflammatory steroids. In the accompanying paper (14), I establish that mononuclear phagocytes contain specific, high affinity receptors for glucocorticoids. In the present paper, I examine the action of glucocorticoids on biochemical functions of monocytes and macrophages cultured in vitro. Mononuclear phagocytes at all stages of maturation are sensitive to glucocorticoids. Production of monocytic and granulocytic colonies from bone marrow precursors and the secretion of elastase, collagenase, plasminogen activator, and nonspecific neutral proteinases by mature macrophages are inhibited by physiological concentrations of glucocorticoids. I have studied macrophages from glucocorticoid-sensitive (rabbit, mouse) and glucocorticoid-insensitive (human, guinea pig) species, and all show similar glucocorticoid-mediated actions, even though they differ in hormone-mediated effects on lymphocytes (1, 3). Because steroid concentrations comparable to those for saturating the high affinity glucocorti-

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* Supported by the U. S. Department of Energy and by grants from the U. S. Public Health Service (AM 14780), the National and New Hampshire Chapters of the Arthritis Foundation, and the Dufault Bequest for Arthritis Research administered by the New Hampshire Chapter of the Arthritis Foundation. A report of part of this work has been published in abstract form (*J. Cell Biol.* 75:191a. Abstr. 1977).

coid binding sites suppress macrophage secretion and colony formation, it is likely that these effects are mediated by specific glucocorticoid receptors.

Materials and Methods

Cells. Human blood monocytes, adjuvant-induced rabbit pulmonary alveolar macrophages, resident mouse peritoneal macrophages, and thioglycollate-elicited mouse peritoneal macrophages were obtained as described in the accompanying paper (14). Pulmonary alveolar macrophages from unstimulated guinea pigs were obtained by lavage procedures similar to those used for rabbits (14, 15).

Cell Culture. Mouse macrophages were cultured in Dulbecco's modified Eagle's medium (DME)[1] supplemented with 10% fetal calf serum (FCS) in 16-mm-diameter multiwell dishes for 24–48 h before experiments. The cultures were washed 4 times with DME. Unless otherwise indicated, the cells were then cultured in DME supplemented with 0.2% lactalbumin hydrolysate (DME-LH) (14, 16). Cortisol and steroids of similar solubility were dissolved at ≅0.1 mM in phosphate-buffered saline, and their concentrations were determined spectrophotometrically (14); less soluble steroids were dissolved in 100% ethanol at ≅0.1 mM and added to cultures to a final concentration of <1% ethanol. Freshly prepared steroid solutions were added to culture media just before use.

Human monocytes (0.5–1.0 × 10^6/16-mm well) were cultured on ^{125}I-labeled fibrin plates (14, 17) in DME + 10% FCS containing 60 μg of soybean trypsin inhibitor (STI)/ml for 24–48 h. The cells were then washed 4 times with DME, and the medium was replaced with DME-LH or DME + 5% dog serum that had been acid-treated and plasminogen-depleted (ATDS-P) (17, 18) with steroids and with or without added dog plasminogen (1–30 nM).

Rabbit and guinea pig alveolar macrophages (0.5–1.0 × 10^6/16-mm dish) were cultured in DME-LH alone for 24–48 h; the medium was then replaced with fresh DME-LH containing steroids as required.

Determination of Bone Marrow Colony Formation. Formation of monocytic and granulocytic colonies (CFU-C) from bone marrow precursor cells was determined by culturing 5 × 10^4 bone marrow cells from adult Swiss mice (CF-1; Charles River Breeding Laboratories, Inc., Wilmington, Mass.) in agar with L-cell colony-stimulating factor (19). Half-maximum concentrations of colony-stimulating activity were used in these experiments, and dexamethasone was added when the plates were poured. Colony number and type were determined at 7 and 14 days of culture; colonies contained at least 50 cells, and clusters contained fewer than 50 cells. Colonies were scored as being granulocytic, monocytic, or mixed.

Metabolic Experiments. Phagocytosis of 1.1-μm-diameter latex particles or sheep erythrocytes coated with 7S immunoglobulin G from rabbit anti-serum to sheep erythrocytes was studied as described previously (13, 16, 20). Metabolism of glucose to CO$_2$ was measured by standard methods (12). For studies of protein synthesis, macrophages were incubated with 5 μCi of [^{35}S]methionine/ml in methionine-free DME, and the amount of cell-associated isotope that was precipitable in 5% trichloroacetic acid was determined.

Enzyme Assays. Lysozyme was assayed with *Micrococcus lysodeikticus* cell walls as substrate (21) and compared with egg white lysozyme standards.

Collagenase was measured with reconstituted ^{14}C-labeled guinea pig skin collagen fibrils as substrate (16). Samples of conditioned medium were activated before assay by treatment with chloromethyl ketone-treated trypsin (TPCK)-trypsin (10 μg/ml, 30 min at 25°C), followed by addition of a fivefold excess of STI (18). 1 U of collagenase activity hydrolyzed 1 μg of collagen fibrils/min at 37°C.

Elastase was assayed quantitatively by measuring release of peptides from [^{3}H]NaBH$_4$-reduced elastin in the presence of the hydrophobic ligand, sodium dodecyl sulfate (SDS) (17). For routine assays, tubes contained 200 μg of elastin and 50 μg of SDS in 100 mM Tris-HCl buffer, pH 7.6, containing 5 mM $CaCl_2$, and 25–200 µl of conditioned culture medium in a final volume of 300 µl. The release of labeled peptides by elastases was linear to ≅40% of the total available radioactive elastin determined by complete hydrolysis with porcine pancreatic elastase. Assays were incubated for 4–24 h as required. 1 U of elastase hydrolyzed 1 µg of elastin/h at 37°C. Elastase was also determined qualitatively by radial diffusion of conditioned medium samples in elastin-SDS-agarose plates (17, 22).

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[1] *Abbreviations used in this paper:* ATDS-P, dog serum that has been acid-treated and plasminogen-depleted; CFU-C, colony-forming units from bone marrow stem cells; DME, Dulbecco's modified Eagle's medium; DME-LH, DME supplemented with 0.2% lactalbumin hydrolysate; FCS, fetal calf serum; K_d, half-maximum concentration; SDS, sodium dodecyl sulfate; STI, soybean trypsin inhibitor; TPCK, chloromethyl ketone-treated trypsin.

Plasminogen activator was measured in several ways. Cells were grown on plates coated with ^{125}I-labeled fibrin, and the release of labeled peptides was monitored (17). Plasminogen activator was also measured in conditioned medium and cell lysates by the fibrin plate assay (17), by direct observation of conversion of ^{125}I-plasminogen to plasmin heavy and light chains (9), and by single-cell assays (13). 1 U of plasminogen activator solubilized 5% of the available ^{125}I-fibrin/h in the presence of plasminogen (≅1 µg/h at 37°C).

For assay of nonspecific neutral proteinases, conditioned culture medium was incubated on ^{125}I-labeled fibrin-coated plates in 50 mM Tris-HCl buffer, pH 7.6, without plasminogen, and the release of radioactive peptides was measured. 1 U of proteolytic activity solubilized 1 µg of ^{125}I-fibrin/h. Protein was determined by the method of Lowry et al. (23).

Unless indicated otherwise, all results are shown as mean ± SD for determinations on three replicate cultures.

Materials. Steroids were purchased from Sigma Chemical Co., St. Louis, Mo., Steraloids, Inc., Pawling, N. Y., or Calbiochem, San Diego, Calif. Cortisone was recrystallized before use. Tissue culture media and supplements were obtained from Grand Island Biological Co., Grand Island, N. Y. Multi-well tissue culture dishes (Costar) were purchased from Microbiological Associates, Walkersville, Md. Elastin from bovine ligamentum nuchae was obtained from Elastin Products, Inc., St. Louis, Mo. Latex particles were purchased from Dow Diagnostics, Inc., Indianapolis, Ind. Anti-serum to sheep erythrocytes was purchased from Cordis Laboratories Inc., Miami, Fla. TPCK-trypsin was purchased from Worthington Biochemical Corp., Freehold, N. J. *M. lysodeikticus*, egg white lysozyme, and bovine fibrinogen were purchased from Sigma Chemical Co. Brewer thioglycollate medium was purchased from Difco Laboratories, Detroit, Mich. [^{3}H]$NaBH_4$ was obtained from New England Nuclear, Boston, Mass. $Na^{125}I$ and [^{35}S]methionine were purchased from Amersham Corp., Arlington Heights, Ill. All other reagents were obtained from standard sources.

Results

General Considerations. In lymphocytes, one of the earliest and most striking effects of glucocorticoids is a change in cellular metabolism; glucose uptake is depressed, ATP economy is affected, and lactate production is decreased (24). During culture of macrophages with glucocorticoids, the cells acidified their culture medium to a reduced extent as a function of glucocorticoid dose. This striking effect was observed in all macrophages (human, mouse, rabbit, guinea pig) cultured at densities high enough to produce a visible pH change in the medium, as monitored by phenol red indicator. The biochemical nature of the inhibition of the pH decrease has not been determined, but this inhibition alone could not account for the decrease in proteinase secretion that occurred. When macrophages are treated with cytochalasin B (3 µM), glucose uptake decreases and the cells fail to acidify culture medium, but secretion of proteinases is not inhibited (13, 25). A small, but consistent decrease in CO_2 production (15–20%) was detected when stimulated mouse macrophages were treated with 10–1,000 nM dexamethasone.

Even after 72 h of treatment, glucocorticoid concentrations of 1,000 nM or less were not toxic to macrophages; this contrasts with the lytic effects of glucocorticoids on lymphocytes (1, 26, 27). Overall protein synthesis measured by incorporation of [^{35}S]methionine was not affected by glucocorticoids (0.1–1,000 nM dexamethasone), although specific changes in the patterns of secreted and cellular proteins were seen (data not shown). Phagocytosis of either latex particles or IgG-coated sheep erythrocytes was not affected by treatment of mouse macrophages with up to 1,000 nM dexamethasone, as observed previously in vitro (13, 28, 29).

Effects of Glucocorticoids on Secretion of Neutral Proteinases by Stimulated Mouse Macrophages

Macrophages elicited in vivo by various inflammatory stimuli secrete neutral proteinases and some other macromolecules at a rate 3–100 times that of unstimulated macrophages (16, 20, 22, 30). Lysozyme, a constitutive secretory product of macrophages and a sensitive indicator of macrophage viability (21), is regulated by a different mechanism than elastase and other proteinases, and its secretion was not affected by glucocorticoids. Thioglycollate-elicited mouse peritoneal macrophages secreted lysozyme at the same rate in controls and cultures treated with dexamethasone (Fig. 1).

GLUCOCORTICOIDS INHIBIT SECRETION OF NEUTRAL PROTEINASES. In contrast to the observations with lysozyme, secretion of elastase, collagenase, and plasminogen activator was inhibited by addition of glucocorticoids to macrophage cultures. Dexamethasone inhibited secretion of elastase by thioglycollate-elicited macrophages (Fig. 1). The inhibition of elastase secretion into the conditioned culture medium did not result in accumulation of the enzyme within the cells; elastase was present in very low quantities in lysates of both control (22) and steroid-treated cells. The decrease in elastase activity could not be attributed to an increased synthesis of an inhibitor of elastase activity in response to dexamethasone; inhibitor could not be detected in mixing experiments with partially purified macrophage elastase and medium from inhibited cultures, and treatment of the steroid-treated cells or conditioned medium with trypsin (10 μg/ml, 30 min at 25°C) did not activate a latent form of the elastase. However, these data do not preclude the possibility that an inactive precursor failed to be secreted or converted to active enzyme as a result of steroid interaction with the cells.

TIME COURSE OF GLUCOCORTICOID INHIBITION. The binding of dexamethasone to macrophages is complete within 30 min at 37°C (14). Steroid effects on plasminogen activator production were not detected in the first 2 h of treatment with 1 nM dexamethasone, but began to be seen at 2 h with 100 nM (Table I). The inhibitory effect was both time and dose dependent. The inhibition was more pronounced at 20 h of treatment than at 5 h. Similarly, inhibition of elastase secretion was more pronounced at 12 h than at 6 h (Table I).

DIFFERENTIAL EFFECTS OF GLUCOCORTICOIDS ON ELASTASE AND PLASMINOGEN ACTIVATOR. Although the secretion of both elastase and plasminogen activator is increased after macrophage stimulation, the two enzymes are regulated independently, as shown by differential effects of colchicine (13, 25). One striking difference was evident when the inhibitory effects of dexamethasone on the secretion of plasminogen activator and elastase were compared (Fig. 2). The concentrations of dexamethasone giving half-maximum inhibition of the secretion of these two enzymes were similar (about 1 nM), but the maximum

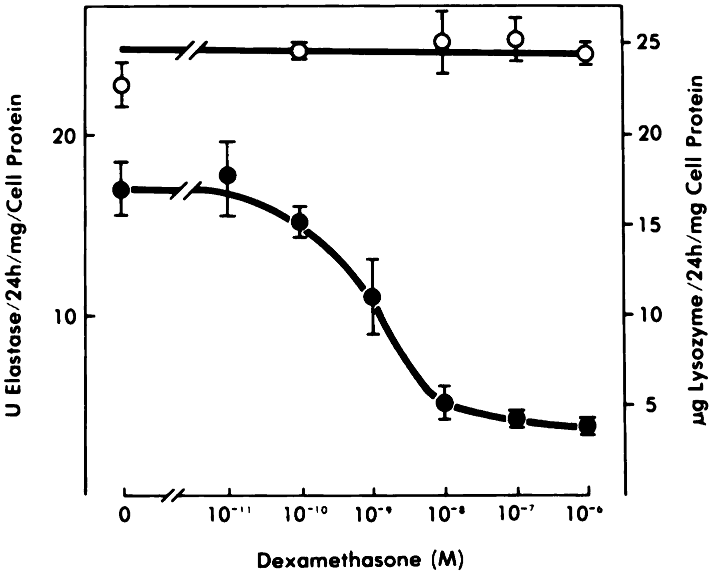

FIG. 1. Comparison of the effects of dexamethasone on the secretion of lysozyme and elastase by thioglycollate-elicited mouse peritoneal macrophages. Macrophages (0.8 × 10^6/2-cm^2 well; 169 μg of cell protein/well) were incubated for 48 h in DME-LH containing various concentrations of dexamethasone. Lysozyme (○) and elastase (●) activities in the conditioned culture media were determined. Results are shown as mean ± SD (n = 3).

TABLE I

Time Course of Dexamethasone Inhibition of Plasminogen Activator and Elastase Secretion by Thioglycollate-Elicited Macrophages

Enzyme	Dexamethasone pretreatment time	Enzyme secreted after dexamethasone treatment	
		1 nM	100 nM
	h	*% control*	
Plasminogen activator*	0	98	81
	5	68	32
	20	51	8
Elastase‡	6	91	64
	12	71	46

* For plasminogen activator experiments, macrophages (2 × 10^5) were plated on ^{125}I-fibrin-coated plates for 24 h, then placed in DME + 5% ATDS-P containing 60 μg of STI/ml and dexamethasone for the pretreatment time indicated. The cultures were then washed and placed in DME + 5% ATDS-P + 1 nM dog plasminogen with the appropriate dexamethasone concentration. The ^{125}I-fibrin hydrolysis was expressed as percentage of plasminogen-dependent degradation by control cultures. Control cultures secreted 43 U of plasminogen activator/10^5 cells/20 h.

‡ For elastase experiments, thioglycollate-elicited macrophages (2 × 10^6/35-mm-diameter plate) were placed in 1 ml of DME-LH containing the appropriate concentration of dexamethasone for 6 or 12 h. Elastase secreted into the medium was expressed as percentage of elastase secreted by cultures without dexamethasone. Values are the means of 3 determinations. Control cultures secreted 2.8 U of elastase in 6 h and 6.8 U in 12 h.

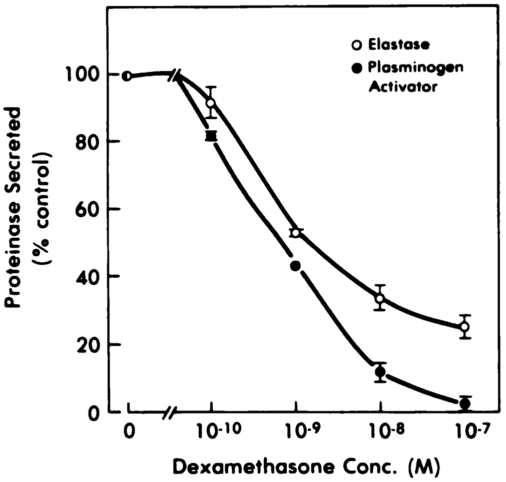

FIG. 2. Comparison of effects of dexamethasone on elastase and plasminogen activator secretion by thioglycollate-elicited mouse macrophages. Macrophages (2 × 10^6/35-mm-diameter well) were cultured in DME + 10% FCS for 48 h, washed 4 times in DME, then cultured in DME-LH in the presence of various concentrations of dexamethasone for 48 h. The enzyme activities in the conditioned medium were measured. Macrophages cultured without dexamethasone secreted 12.6 ± 1.2 U of elastase and 117 ± 18 U of plasminogen activator in 48 h. Values represent the mean ± SD (n = 3); elastase (○); plasminogen activator (●). Conc., concentration.

inhibition of plasminogen activator secretion (97–100%) was greater than that for elastase secretion (65–80%). Inhibition of elastase secretion was not complete, even after 120 h in the presence of 1 μM dexamethasone. Although it is possible that the elastase activity was due to the presence of two enzymes, one of which was glucocorticoid sensitive and the other insensitive, this is unlikely. The residual 20–35% of elastase secreted during steroid treatment had the same inhibitor profile as enzyme secreted by control cells. This modulation, rather than total inhibition or induction of cellular proteins, is characteristic of the effects of glucocorticoids in many other systems (1, 6, 24).

GLUCOCORTICOID INHIBITION OF MACROPHAGE SECRETION IS REVERSIBLE. After treatment with dexamethasone for 24 h, the secretion of elastase was restored to at least 80% of control values during the next 24 h. For plasminogen activator, reversal of the inhibitory effect of 10 nM dexamethasone was detected within 2 h after removal of the drug by assay on fibrin plates, by single-cell assays with a casein overlay in the presence of plasminogen, and by conversion of ^{125}I-plasminogen to ^{125}I-plasmin.

INHIBITION OF MACROPHAGE SECRETION OF PROTEINASE IS A SPECIFIC EFFECT OF GLUCOCORTICOIDS. Only glucocorticoids inhibited secretion of proteinases by macrophages. Dexamethasone, cortisol, and corticosterone inhibited secretion of elastase and collagenase, whereas the anti-glucocorticoids (cortexolone and progesterone), a glucocorticoid analogue (11-epicortisol), and the sex steroids had no inhibitory effects (Table II).

PROGESTERONE ANTAGONIZES THE GLUCOCORTICOID INHIBITION OF MACROPHAGE SECRETION. Progesterone has no glucocorticoid activity, although it competes

TABLE II
*Effects of Various Steroids on Secretion of Elastase and Collagenase by Thioglycollate-Elicited Mouse Macrophages**

Steroid	Concentration	Elastase secreted	Collagenase secreted
	nM	*U*	*mU*
None	–	18.3 ± 3.6	119 ± 31
Dexamethasone	10	n.d.	59 ± 7
	1,000	4.1 ± 0.2	0
Cortisol	100	12.2 ± 0.2	77 ± 35
	1,000	8.5 ± 0.7	22 ± 19
Corticosterone	100	9.6 ± 0.1	46 ± 23
	1,000	5.9 ± 0.7	0
Cortexolone	100	15.4 ± 2.0	n.d.
	1,000	12.9 ± 0.2	n.d.
Progesterone	1,000	19.7 ± 1.3	96 ± 26
Cortisone	1,000	n.d.	104 ± 42
11-Epicortisol	100	21.0 ± 4.5	n.d.
Dihydrotestosterone	200	16.3 ± 1.1	n.d.
Estradiol	500	20.2 ± 0.9	127 ± 41

* Macrophages (3.2×10^6) were plated in DME + 10% FCS for 32 h, washed 4 times with DME, then placed in 1.2 ml of DME-LH with steroids for 66 h. Collagenase was assayed after media were dialyzed and lyophilized, reconstituted at 0.1 of original volume, and then activated with trypsin (n.d., not determined). Values are shown as mean ± SD (n = 3).

with glucocorticoids for receptor binding (14). Alone, progesterone had no effect on elastase secretion; in combination with either dexamethasone or cortisol, however, progesterone blocked the glucocorticoid-mediated inhibition of elastase secretion (Fig. 3).

The Effect of Serum on Secretion of Plasminogen Activator and Elastase. Secretion of both plasminogen activator and elastase by thioglycollate-elicited mouse macrophages was detected in the presence of 5-10% ATDS-P. Serum contains endogenous cortisol and progesterone, but there was no significant deviation in dose responsiveness of secretion between dexamethasone with serum and dexamethasone without serum. Therefore, the steroids in dilute serum contributed little to the modulation of macrophage secretion.

Effects of Glucocorticoids on Mouse Monocytic Precursor Cells and on Unstimulated Peritoneal Macrophages

DEXAMETHASONE INHIBITS CELL DIVISION DURING FORMATION OF MONOCYTIC AND GRANULOCYTIC COLONIES FROM MOUSE BONE MARROW PRECURSOR CELLS IN VITRO. The anti-inflammatory effect of glucocorticoids is reflected by a decrease in mononuclear phagocytes in inflammatory exudates, and by a severe monocytopenia (1-6); it has been suggested that monocyte production is diminished by an inhibition of promonocyte division (5). Although glucocorticoid receptors have been studied only in monocytes and in later stages of mononuclear phagocyte differentiation (14), the earliest accessible stage of macrophage differentiation that can be studied in vitro is the formation of monocytic and granulocytic colonies from committed bone marrow precursor cells.

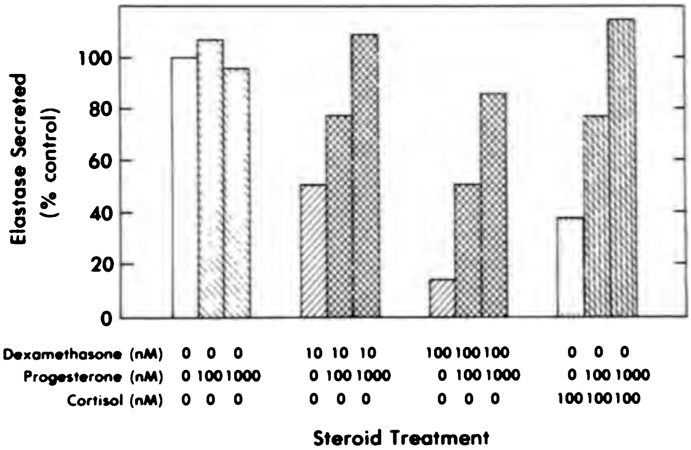

FIG. 3. Antagonism by progesterone of the glucocorticoid inhibition of elastase secretion by thioglycollate-elicited mouse macrophages. Macrophages (2×10^6) were cultured for 24 h in DME + 10% FCS, washed 4 times in DME, then placed in DME-LH containing dexamethasone, progesterone, or cortisol at various concentrations for 48 h; elastase activity of the conditioned media was then measured. Control macrophages incubated in DME-LH alone secreted 16.3 ± 2.7 U of elastase in 48 h. Values are means of three replicate cultures.

Dexamethasone reduced the number of CFU-C that formed in vitro (Table III). After 7 days of incubation, 50% inhibition of CFU-C was seen at 20 nM dexamethasone, and very few colonies were found at 100 nM or higher concentrations. However, many clusters containing fewer than 50 cells were seen at several concentrations of the steroid, suggesting that the decreased number of colonies was due to a diminution in the rate of cell division, rather than to the lysis of the precursor cells. By 14 days of culture, a maximum of 50% inhibition of CFU-C formation was observed at the highest dexamethasone concentration (1,000 nM). The cultures with 1,000 nM dexamethasone contained more clusters of cells than control cultures, suggesting that the number of colonies in the treated cultures would approach the control level after further incubation. Both monocytic and granulocytic colonies were seen in control and dexamethasone-treated cultures, and the glucocorticoid decreased the two populations proportionately (Table III). Because cultures of bone marrow cells were heterogeneous, it could not be determined if the glucocorticoids acted on the stem cells directly or on accessory cells required for differentiation and colony formation in vitro.

GLUCOCORTICOIDS INHIBIT SECRETION BY RESIDENT MOUSE PERITONEAL MACROPHAGES. Unstimulated macrophages secrete very small quantities of plasminogen activator, collagenase, or elastase (16, 20, 22, 30); their secretion of lysozyme is comparable to that of stimulated macrophages (21) (1×10^6 macrophages secreted 2.2 μg of lysozyme, 1.2 U of elastase, and 4.3 U of plasminogen activator per 24 h). The ability of steroids to modulate these basal levels of secretion was examined next. Extracellular secretion of elastase and plasminogen activator by resident macrophages was inhibited 79–97% by glucocorticoids (100 nM dexamethasone, triamcinolone acetonide, and cortisol)

TABLE III

*Effect of Dexamethasone on Monocytic and Granulocytic Colony (CFU-C) Formation in Vitro**

Dexamethasone	Incubation time	CFU-C type		Total CFU-C (per 5 × 10^4 cells)	Cell clusters (per 5 × 10^4 cells)
		Monocytic	Granulocytic		
nM	*days*	%			
0	7	9 ± 2	62 ± 5	54 ± 7	19
	14	8 ± 2	65 ± 6	107 ± 17	22 ± 2
0.1	7	16 ± 5	60 ± 3	52 ± 6	19 ± 2
	14	9 ± 1	61 ± 6	112 ± 13	28 ± 6
1.0	7	12 ± 2	63 ± 6	56 ± 6	24 ± 3
	14	10 ± 2	61 ± 5	108 ± 7	27 ± 4
10	7	11 ± 3	74 ± 9	37 ± 8	44 ± 2
	14	7 ± 2	73 ± 3	90 ± 15	41 ± 4
100	7	8 ± 4	92 ± 4	5 ± 1	20 ± 2
	14	10 ± 3	74 ± 2	71 ± 9	48 ± 10
1,000	7	0	100	1 ± 1	14 ± 8
	14	13 ± 5	73 ± 3	54 ± 9	43 ± 9

* Mean ± SE (3 experiments, each in duplicate). CFU-C were designated as monocytic, granulocytic, or mixed. Percentage of mixed CFC-C is not shown. Colonies and clusters were classified as described in Materials and Methods.

but not by other steroids (100–1,000 nM cortisone, progesterone, cortexolone, dihydrotestosterone, 11-epicortisol), whereas lysozyme secretion was not affected by any of the steroids. The concentration of dexamethasone giving half-maximum inhibition of elastase secretion (2 nM) (Fig. 4) was the same as the concentration that gave half-maximum saturation of the high affinity glucocorticoid receptor in these cells (2.1 nM) (14). Cortisol was less effective as an inhibitor, reflecting its lower binding affinity for the receptor (14). Cortisone, which does not bind to receptors, reproducibly showed a small inhibitory effect on elastase secretion, but it is possible that macrophages convert cortisone to cortisol at a low rate. These results were qualitatively similar to those obtained with stimulated macrophages.

Effects of Glucocorticoids on Mononuclear Phagocytes from Other Species

Significant species differences exist in the responses of cells to glucocorticoids (1, 3) and in secretion of proteinases. Accordingly, macrophages from three other species were examined to study the general applicability of the results of glucocorticoid treatment of mouse cells. Human, rabbit, and guinea pig macrophages all contained specific receptors for glucocorticoids (14).

HUMAN BLOOD MONOCYTES. Monocytes cultured on ^{125}I-fibrin layers catalyze plasminogen-dependent fibrinolysis at low rates (30). The production of plasminogen activator was inhibited by addition of dexamethasone to the cultures. As shown in Table IV, at 20 h of treatment the release of ^{125}I-labeled peptides was reduced to 42% of control by 1 nM dexamethasone, and to <10% of control by 100 nM; estradiol, dihydrotestosterone, and progesterone had no effect on production of plasminogen activator. This pattern of steroid effects correlates with the observed specificity and avidity of glucocorticoid binding to monocytes (14).

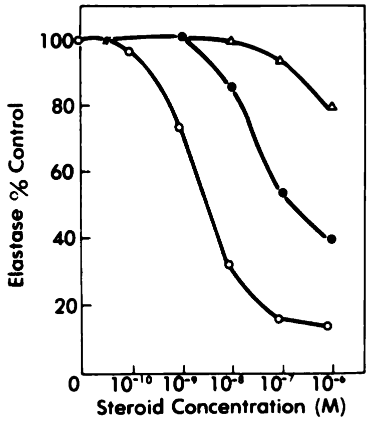

FIG. 4. Dose dependence of glucocorticoid inhibition on elastase secretion by resident mouse peritoneal macrophages. Macrophages (6×10^5/2-cm^2 well) were incubated with the steroids in DME-LH for 48 h, and then elastase activity in the conditioned media was determined. Control cells secreted 288 mU of elastase in 48 h. Wells received either dexamethasone (○), cortisol (●), or cortisone (△). Values are means of three wells.

TABLE IV

Effect of Various Steroids on Production of Plasminogen Activator by Human Monocytes

Added steroid	Concentration	^{125}I-fibrin degradation*
	nM	*% control*
None	–	100
Dexamethasone	1	41.3
	100	8.1
Estradiol	100	109.9
Dihydrotestosterone	100	94.8
Progesterone	100	105.2

* Monocytes ($\cong 5 \times 10^5$) were cultured on ^{125}I-fibrin layers in DME containing 5% ATDS-P with the steroid. The plasminogen-dependent fibrinolysis was measured after 20 h. Values are shown as mean of 3 replicate cultures. Control cultures lysed 17.2% of the available ^{125}I-fibrin ($\cong 1$ U of plasminogen activator).

RABBIT ALVEOLAR MACROPHAGES. Macrophages from the granulomatous lungs of rabbits secreted several proteolytic enzymes that hydrolyzed collagen, fibrin, and elastin, and activated plasminogen at neutral pH. Two key features of dexamethasone inhibition of collagenase, fibrinolytic proteinase, elastase (Fig. 5), and plasminogen activator became apparent. Although the half-maximum concentration (K_d) for dexamethasone binding to the alveolar macrophages was 1-3 nM, the concentrations of dexamethasone producing 50% inhibition of proteinase secretion were as low as 0.05-0.1 nM, and the effects were seen at as little as 0.01 nM. Secondly, the four distinct proteolytic activities had different responses to the inhibitory effects of dexamethasone. The elastase (Fig. 5 A) was inhibited to a lesser extent than the collagenase, neutral proteinase (Fig. 5 B), and plasminogen activator (data not shown), even at 1

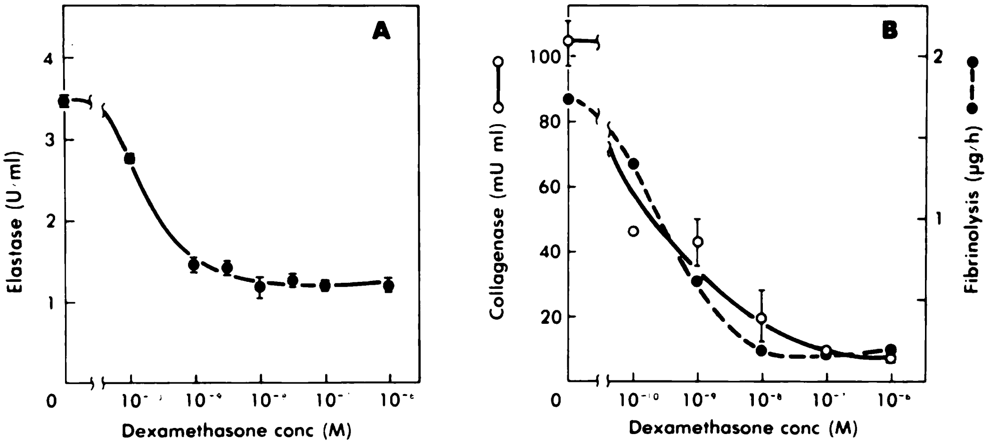

FIG. 5. Effect of dexamethasone on secretion of proteinases by adjuvant-elicited rabbit alveolar macrophages. Macrophages were plated at 1.4 × 10^6 cells/2-cm^2 well. (A) Secretion of elastase was studied by incubating macrophages in DME-LH containing dexamethasone for 96 h; conditioned culture media were then assayed for activity with ^3H-labeled elastin-SDS as substrate. Values are shown as mean ± SD (n = 3). (B) Secretion of collagenase and fibrinolytic neutral proteinase was examined by performing enzyme assays on conditioned culture media after 48 h incubation with dexamethasone. Values are shown as mean ± SD (n = 3). Collagenase activity (O- - -O) was determined with radioactive collagen fibrils as substrate. Neutral proteinase activity (●——●) was determined with ^{125}I-labeled fibrin as substrate. Conc., concentration.

µM dexamethasone. Similarly, with mouse exudative macrophages, dexamethasone inhibited plasminogen activator secretion to a greater degree than elastase (Fig. 3).

GUINEA PIG MACROPHAGES. An elastase-like proteinase was easily measured in the conditioned medium of guinea pig lung macrophages. Exposure of these macrophages to dexamethasone (10 nM) decreased production of this proteinase to 57% of control values. These inhibitory effects contrast with the relative insensitivity of guinea pig lymphocytes to the catabolic effects of glucocorticoids (1).

Discussion

Glucocorticoids are allosteric effectors which regulate differentiation and modulate physiological responses. In macrophages and other target cells, the binding of the glucocorticoid to its "cytosol" receptor induces an "activated" state in the steroid receptor, which then acquires affinity for chromosomal sites (14, 31, 32). The steroid-receptor interaction with the genome triggers the biological response characteristic of the target tissue. Although the receptor proteins are present at relatively low intracellular concentrations (≅5,000 molecules per macrophage [14]), the behavior of the receptors can be followed selectively because of their high specific affinity (K_d = 1 nM) for the tritium-labeled hormone. The selectivity of the biological response may occur because productive gene activation is achieved only when receptor molecules occupy multiple sites in the same genetic region (32). In the macrophage, genetic loci regulated by glucocorticoids have not been identified.

In this paper I have explored the action of glucocorticoids on key physiological functions of mononuclear phagocytes in culture. The most accessible effect of glucocorticoids on macrophage functions are inhibitory or catabolic, but glucocorticoids can be anabolic as well (31). In general, the molecular mechanisms for the catabolic effects of glucocorticoids are not as well understood as are the anabolic or inductive effects. Glucocorticoids did not produce measurable changes in overall RNA synthesis of macrophages, although specific effects probably occurred. Overall protein synthesis by mouse macrophages was not affected by glucocorticoids, but some new cellular and secreted proteins appeared, and other proteins decreased with dexamethasone treatment (unpublished data). Low concentrations of dexamethasone (0.4–40 nM) induce angiotensin-converting enzyme in rabbit alveolar macrophages (33).

Steroid-mediated effects occurred at all stages of macrophage development tested, from the differentiation of committed bone marrow stem cells into monocytes to the metabolic and secretory activity of mature macrophages responding to inflammatory stimuli. To assess the general significance of the results described in this paper it is important to distinguish effects of glucocorticoids that are responses of eukaryotic cells in culture from those that are specific to macrophages. First, the proliferation of many cell types is regulated by glucocorticoids (26, 27, 31); however, macrophage differentiation shows some specificity of response both in vitro and in vivo. The rate of formation of macrophage colonies from bone marrow stem cells was inhibited by dexamethasone. In vivo the number of circulating monocytes decreases after steroid treatment (4, 5). Low concentrations of glucocorticoids also inhibit granulopoiesis as shown in this study and previously (34, 35), but potentiate erythropoiesis (36). Second, plasminogen activators are produced by many cells, including monocytes and macrophages (13, 18, 20, 30, 37). Inhibition of plasminogen activator synthesis and secretion by glucocorticoids at physiological concentrations is not a specific response of macrophages, but a common feature of the regulation of this enzyme in many cell types (13, 38, 39). Third, collagenase secretion by macrophages and by synovial cells, fibroblasts, and skin explants is inhibited by low concentrations of dexamethasone and other corticosteroids (18, 40, 41), which probably accounts for the slower rate of collagen degradation in granulomas in vivo in the presence of these steroids (42). Thus, the regulation of collagenase production by glucocorticoids is not unique to macrophages. Fourth, secretion of macrophage elastase was inhibited by glucocorticoids. Because secretion of macrophage elastase appears to be a specific property of the mononuclear phagocyte series (unpublished data) its inhibition by glucocorticoids is a specific response of the macrophage. Finally, angiotensin-converting enzyme is induced in macrophages by glucocorticoids (31). This enzyme is produced by a number of cell types and its regulation by glucocorticoids in these cells has not been established. Although few of these responses to glucocorticoids are specific to macrophages, taken together, the spectrum of responsive proteins provides a distinctive profile or domain for the macrophage target cell.

The inhibitory effects on proteinase secretion described could be either direct effects of glucocorticoids on genetic loci that control synthesis and processing of these proteins by macrophages, or indirect actions on loci that determine effector molecules, which then regulate proteinase secretion by macrophages. Prostaglandins, which are mediators of inflammation, are secreted by stimulated macrophages (43–45), and may modulate secretion of collagenase by guinea pig macrophages (43). In various cell types, production of prostaglandins is inhibited by glucocorticoids (41, 46), which block the release of arachidonic acid from phospholipids (47). It is possible that prostaglandins mediate some of the effects of steroids on macrophages; however, it is unlikely that they account for all of the effects of glucocorticoids. Additions of exogenous prostaglandins or arachidonic acid do not overcome the inhibition by dexamethasone, and indomethacin, an inhibitor of prostaglandin synthetase, does not have the pleiomorphic effects of glucocorticoids on physiological functions of macrophages (unpublished data). Other regulatory molecules that influence macrophage functions may also be mediators of glucocorticoid action. Lymphokines (44, 48), cyclic nucleotides (13), or proteinases secreted by the macrophages (48, 49) may mediate the inhibitory effects of glucocorticoids. Further investigation is necessary to assess the relative contribution of these factors to the glucocorticoid responsiveness of macrophages.

Because products of other cells, particularly lymphocytes, may modulate macrophage functions such as secretion (1, 8), it is important to establish whether or not glucocorticoids act directly on mononuclear phagocytes. Although macrophage populations are often considered homogeneous, the primary cultures are contaminated to varying degrees by small numbers of mesothelial cells, fibroblasts, and adherent lymphocytes (50, 51). In vivo, lymphocytes may be glucocorticoid target cells for functions expressed by macrophages (1, 8). In the overtly heterogeneous populations of bone marrow, this question remains unresolved. In the more homogeneous cultures of peritoneal and alveolar macrophages, it is likely that the glucocorticoids acted directly, because populations of macrophages contaminated to varying degrees by nonadherent cells did not respond differently to the steroids. This interpretation is supported by the observation of similar inhibitory effects of glucocorticoids on proteinase secretion with a homogeneous line of macrophages (P388D1), derived from a tumor (52).

In view of the inhibitory effects of glucocorticoids on macrophages at subphysiological concentrations (e.g., Fig. 5), the degree to which glucocorticoid-sensitive functions are expressed in vivo in the presence of potentially inhibitory concentrations of steroids is of interest. Effective cortisol concentrations in vivo are modulated by the plasma-binding globulin, transcortin (53), and by progesterone, an antiglucocorticoid (31, 54). In low concentrations of serum, secretion of plasminogen activator and elastase by thioglycollate-elicited macrophages and formation of monocytic CFU-C were not affected, and the serum steroids did not interfere with the inhibitory effects of dexamethasone. Effective inhibition of collagenase secretion by 1 nM dexamethasone in 10% FCS has been reported in another cell system (41). Progesterone antagonized the inhibitory effects of exogenous glucocorticoids on proteinase secretion, as reported here, and on granulopoiesis (34). It is probable that physiological concentrations of glucocorticoids, in the presence of physiological concentrations of progesterone, may not suppress these macrophage functions effectively.

Although receptor proteins in macrophages have a binding affinity for dexamethasone of $\cong 1$ nM, glucocorticoid effects were observed at concentrations as low as 0.01 nM in the present work, and other glucocorticoid responses at subphysiological concentrations have reported in macrophages (33, 34) and in other cells (40, 41). Thus, undetected specific sites of even higher affinity for glucocorticoids may exist, as proposed for estradiol receptors (55). An alternative explanation is that the specific effects are initiated when only a few hormone-receptor complexes have bound to the genome at these low concentrations. In most early studies of glucocorticoid effects on macrophages, pharmacological concentrations of steroids were used to achieve effects (4, 5, 8, 9, 11, 29, 35, 56, 57). Because analysis of steroid receptors reveals no low affinity receptors specific for glucocorticoids (14), many of these effects are probably not receptor-mediated.

Products of macrophage secretion are regulated by diverse mechanisms. Lysozyme, a product that is secreted by macrophages in all states of stimulation (21), was not affected by glucocorticoids. Plasminogen activator, collagenase, and elastase are apparently secreted coordinately after macrophage stimulation in vivo and in vitro (13, 16, 20–22) but are, in fact, regulated by independent mechanisms. Plasminogen activator secretion is inhibited by colchicine (13) and prostaglandins (unpublished data), whereas elastase and collagenase secretion is stimulated by these drugs (25, 43, and unpublished data). Although secretion of these three proteinases was inhibited by glucocorticoids, differential sensitivities were observed. In general, plasminogen activator secretion was inhibited to a greater extent (>95%) than was elastase secretion (60–80%). These data support classification of these proteinases into separate regulatory groups sharing a few common pathways. Macrophages have been shown to secrete several products in addition to lysozyme and the neutral proteinases. Secretion of endogenous pyrogen (57) and lysosomal hydrolases (58) may also be inhibited by corticosteroids. The pathways for secretion of some complement components and proteinases have some similar regulatory sequences (59), and it is possible that complement proteins are also modulated by glucocorticoids. Macrophages secrete both proteinases and proteinase inhibitors, e.g., α_2-macroglobulin (60). The regulation of α_2-macroglobulin production is not established, but it is unlikely that changes in its secretion by glucocorticoids could produce the observed inhibition of proteinase secretion.

Some metabolic functions were affected by glucocorticoids in the mononuclear phagocytes tested from four species (human, mouse, rabbit, guinea pig); it is not clear, however, whether the spectrum of macrophage response is similar in all species and in all tissues. Although the concentration of glucocorticoid receptors decreased with increasing state of macrophage differentiation or activation (14), monocytes, resident macrophages, and exudative macrophages were all sensitive to glucocorticoids. Similar observations in lymphocytes suggest that increases in glucocorticoid receptor concentrations do not increase steroid sensitivity (61). Mononuclear phagocytes treated with glucocorticoids phenotypically appear to be less differentiated or stimulated than untreated macrophages in their secretion of proteinases and their metabolism. Further studies with glucocorticoids as probes may allow us to define the pathways of macrophage differentiation.

Summary

The effects of glucocorticoids on biochemical functions of macrophages from man, mouse, rabbit, and guinea pig were examined. Secretion of plasminogen activator by human peripheral blood monocytes was decreased by 50% with 1 nM dexamethasone. Differentiation of murine monocytic and granulocytic colonies in agar from bone marrow precursors was decreased by 50% at 7 days with 20 nM dexamethasone. Secretion of elastase, collagenase, and plasminogen activator by resident and thioglycollate-elicited mouse peritoneal macrophages was decreased by dexamethasone, cortisol, and triamcinolone acetonide (1-1,000 nM), but not by progesterone, estradiol, and dihydrotestosterone (1,000 nM); in contrast, secretion of lysozyme was not affected by glucocorticoids or other steroids. The inhibition of macrophage secretion by dexamethasone was both time and dose dependent. Effects were detected within 1-6 h after addition of the glucocorticoids, became maximum by 24 h, and were reversed during a similar time period after removal of the hormones. The extent of inhibition of macrophage secretion increased with increasing glucocorticoid concentration.

Half-maximum inhibition of secretion of elastase, collagenase, and plasminogen activator was seen at dexamethasone concentrations (1-10 nM) similar to those that half-saturated the specific glucocorticoid receptors in these cells. At high concentrations of dexamethasone (100-1,000 nM) the secretion of plasminogen activator was inhibited to a greater extent (>95%) than the secretion of elastase (60-80%). Progesterone alone had no effect on secretion, but it blocked the inhibitory effects of dexamethasone and cortisol. Secretion of collagenase, neutral proteinases, and plasminogen activator by elicited rabbit alveolar macrophages was inhibited with glucocorticoids (0.1-100 nM) but not with progesterone or sex steroids. Secretion of a neutral elastinolytic proteinase by guinea pig alveolar macrophages was also inhibited by dexamethasone. These data support the regulatory role of glucocorticoids on macrophage functions at physiological concentrations.

I thank Jennie Chin for her technical assistance, Ingrid Vander Heiden for her assistance with the animals, and Mary Maloney and Georgina Dunn for performing the CFU-C assays. This work was initiated while I was a visiting faculty member in the Connective Tissue Disease Section, Department of Medicine, Dartmouth Medical School, Hanover, N. H. I am indebted to Professor E. D. Harris, Jr., for his encouragement and support, and I thank Professor A. Munck for his stimulating discussion about some aspects of this work.

Received for publication 27 January 1978.

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THE HUMAN COMPLEMENT SYSTEM IN THROMBIN-MEDIATED PLATELET FUNCTION*

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The role of complement in blood coagulation is far from clear. A clotting abnormality was reported (1, 2) in rabbits deficient in the sixth component of complement (C6), however, no clotting abnormality was detected in a human patient deficient in the same component (3). When platelets were incubated in fresh human serum, C3 and C5–C9 complexes were demonstrated on the platelet membrane as well as C5–C9 complexes in the fluid phase of the reaction mixture (4). Further, retraction and lysis of thrombin-induced blood clots were inhibited by antiserum to C3 and C4 (5). We have previously reported (6) that complement-dependent ultrastructural lesions were visualized on the platelet surface subsequent to their incubation with thrombin and complement.

In the present study, thrombin-mediated specific uptake of C3 and C5 was demonstrated by uptake of radiolabeled components and was visualized ultrastructurally utilizing ferritin-conjugated monospecific antiserum to each of the two components. To develop these studies further, the role of complement in thrombin-induced platelet function was investigated. It was found that whereas complement was not essential for thrombin-mediated platelet aggregation and release of serotonin, these two activities were much enhanced in the presence of complement. When the nature of the complement interaction was determined, it was found that components C3, C5, C6, C7, C8, and C9 were essential for this enhanced reactivity; however, these six components in the absence of any previously described C3 convertase were the only components of the complement system that were required. Evidence is presented identifying a new pathway of activation of complement—one that is dependent on the presence of thrombin and the platelet membrane and enters the known complement sequence at the C3 stage.

Materials and Methods

Preparation of Washed Platelets. A suspension of human platelets was prepared using the Ardlie buffer system as previously described (7) with the following modification. Because the pH of the Ardlie buffers was found rapidly to increase, the sodium bicarbonate in each buffer was replaced by Tris (hydroxymethyl) amino methane (Trizma base) at the same molarity, namely, 0.012 M. Under these conditions, the pH of the buffers remained constant at pH 7.3 throughout the experiment.

Preparation of Aluminum Hydroxide-Absorbed Serum. Fresh human serum was absorbed with aluminum hydroxide as previously described (6) under conditions that were shown to remove

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* Supported by the National Institutes of Health through a specialized Center for Thrombosis grant (HL 18828) and the Arnold R. Krakower Foundation.

prothrombin but not to inactivate complement. Serum was considered to be prothrombin free if, after incubation with fibrinogen (1.5 mg/ml) for 48 h, no clot was formed. Assays were performed to measure activity of whole component and components of both the classic and alternative mechanism as previously described (6).

Preparation of Complement Components and Reagents. C3(8), C4(9), C5(8), C6, C7(10), C8(11), and C9(12) were prepared by methods described earlier. In addition, for some experiments purified complement components prepared by Cordis Laboratories, Inc. (Miami, Fla.) were utilized. C3a and C5a were produced by trypsin treatment of the parent molecule and molecular sieve chromatography (13, 14). Aluminum hydroxide ($Al[OH]_3$)-absorbed serum was further treated with potassium thiocyanate (KSCN)[1] under conditions known to inactivate C3, C4, and C5(15). In reconstitution experiments, 25 μg C3, 17 μg C4, and 3 μg C5 were added either singly or in combination to 10 μl of $Al(OH)_3$-absorbed KSCN-treated serum. A reagent that contained C3, C5, C6, and C7 and not C8 and C9 was also prepared (11).

Human serum totally deficient in C2 was kindly supplied by Doctors Kunkel and S. M. Fu (The Rockefeller University, New York). Factor B-depleted serum was prepared by heating fresh human serum at 50°C for 30 min (16). A serum reagent lacking C2 and Factor B was prepared by heating the C2-deficient serum at 50°C for 30 min. Each serum was absorbed with aluminum hydroxide as described above. In the case of the heated sera, the absorption with aluminum hydroxide was performed before the heating step.

Monospecific antisera to C2, C3, C5, C8, and C9 were prepared in rabbits by injection of the purified protein into the popliteal lymph nodes followed a month later by an intramuscular injection of the same protein (17). The antisera were extensively tested immunochemically for specificity and if they were found not to be monospecific, they were appropriately absorbed. Monospecific anti-human serum albumin and anti-Factor B were purchased from Behring Diagnostics, Inc. (Woodbury, N.Y.).

Platelet Aggregation. Assays for platelet aggregation were performed in a Payton dual channel aggregometer using a Riken Denshi recorder (Payton Associates Inc., Buffalo, N.Y.). Platelets were suspended at 200,000/μl in Ardlie II buffer in which bicarbonate had been replaced with Tris (see above). All reagents added to the platelets were dialyzed before use vs. cacodylate buffer pH 7.4, a buffer shown to be optimal for aggregation of washed platelets (18). Highly purified human thrombin (2.05 U/μg) was prepared and kindly supplied by Dr. John Fenton, New York State Department of Health, Albany, N.Y. 0.1 U in 10 μl was added to 0.3 ml washed platelets.

Release of ^{14}C-Serotonin. Platelets suspended in plasma were labeled with ^{14}C-serotonin by the method of Valdorf-Hansen and Zucker (19). The radiolabeled platelets were then washed in the usual way. For the experiment, 0.3 ml platelet suspension was utilized to which 10 μl aliquots of various reagents were added. Aggregation was recorded for 5 min, then the tube was centrifuged and the supernate removed. ^{14}C was counted in both the supernate and the cell button in a Packard Liquid Scintillation counter (Packard Instrument Co., Inc., Downer's Grove, Ill.) and percent release was calculated.

Release of Lactic Dehydrogenase (LDH). LDH release was determined by the method of Wroblewski and LaDue (20). 1 U was expressed as decrease in OD at 340 μm of 0.001/min per ml. Release of LDH during incubation periods from 1 min to 2 h was determined.

Ferritin Conjugation of Antisera. Monospecific antisera to C3 and C5 were conjugated with ferritin by the method of Tawde and Ram (21). Free ferritin and unconjugated γ-globulin were separated from the conjugated γ-globulin by pevikon block electrophoresis (22).

Preparation of Platelets for Electron Microscopy. Complement was activated on the platelet surface by the methods described in detail below. After incubation with complement, the platelets were washed three times with saline. The platelet button was then suspended in a dilution of ferritin-conjugated antibody and incubated at 37°C for 30 min. After this incubation the platelets were washed three times in saline, then divided into two aliquots. One aliquot was then washed three times in a 1/10 dilution of saline buffered to pH 6.5. After the first wash step at this low ionic strength, the platelets were frozen and thawed twice to lyse them, and the unstained platelet membranes were viewed in a Philips 301 Electron Microscope (see Fig. 1). The second aliquot of platelets was embedded, sectioned, and stained in the usual way and then viewed under the electron microscope (see Fig. 2).

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[1] *Abbreviations used in this paper:* DFP, diisopropyl fluorophosphate; KSCN, potassium thiocyanate; LDH, lactic dehydrogenase.

Radiolabeling of Complement Components. C3 and C5 were labeled with ^{125}I by the method of McConahey and Dixon (23). The specific activity of C3 was approximately 200,000 cpm/μg; and of C5 was approximately 60,000 cpm/μg.

Activation of Complement on the Platelet Surface. The serum concentration of C3 was assumed to be 1.3 mg/ml and of C5 80 μg/ml. For each experiment either 25 μl of ^{125}I-C3 containing 20.5 μg of protein or 25 μl of ^{125}I-C5 containing 5.25 μg was added to 0.25 ml of undiluted human serum. Complement was activated on the platelet surface by (*a*) the classic mechanism, (*b*) the alternative mechanism, and (*c*) by thrombin:

(*a*) Activation of complement by the classic mechanism: 0.5 ml of a platelet suspension at 1.5 × 10^9/ml was incubated with 0.25 ml serum containing an isoantibody to a nonidentified platelet antigen and 0.25 ml of fresh serum containing either ^{125}I-labeled C3 or C5. After incubation at 37°C for 90 min, the tubes were spun and the cell button was washed five times with saline before counting.

(*b*) Activation of complement by the alternative mechanism: 0.25 ml of the platelet suspension was mixed with 0.25 ml of inulin at 10 mg/ml. The mixture was centrifuged and the supernate was discarded. To the inulin-platelet mixture was added 0.25 ml serum containing either ^{125}I-C3 or ^{125}I-C5. After incubation at 37°C for 90 min, the tubes were centrifuged and the cell button was washed five times with saline.

(*c*) Activation of complement with thrombin: 0.5 ml of the platelet suspension was mixed with 0.25 ml serum containing 0.25 U of thrombin and ^{125}I-C3 or C5. After incubation at 37°C for 90 min, the tubes were centrifuged and the cell button was washed five times with saline.

In an experiment to determine the role of the platelet in the activation of complement by thrombin, a similar method was used except that the platelets were replaced by either human erythrocytes at 5 × 10^8/ml or leukocytes at 5 × 10^7/ml. The leukocyte preparation was the supernate from dextran-sedimented defibrinated whole blood.

In each of the above experiments the negative control for nonspecific uptake of radiolabeled complement component was supplied by a similar reaction mixture containing 0.01 M EDTA. In each case this figure was subtracted from the total uptake to provide specific uptake.

Inhibition of Aggregation and Release of Serotonin by Monospecific Antisera. Monospecific antisera to C2, C3, C5, C9, Factor B, and human serum albumin were utilized. 0.2 ml of each antiserum was applied to a *O*-triethylaminoethyl-cellulose column equilibrated with phosphate buffer pH 8.0 and 0.0175 M. The γ-globulin fraction was eluted with the same buffer. The fraction containing the peak of γ-globulin was used in an unconcentrated form. 0.3 ml was incubated at 37°C for 15 min with 20 μl of antibody. Subsequent to this incubation, the tube containing the antibody-platelet mixture was transferred to the platelet aggregometer, and 10 μl samples of various complement components or reagents were added (see Results). The anti-human serum albumin was used in each experiment as the negative control.

Treatment of Thrombin with Diisopropyl Fluorophosphate (DFP). DFP at a final concentration of 2 × 10^{-3} M was added to thrombin at 100 U/ml. The mixture was allowed to stand at 0°C for 30 min then was dialyzed extensively vs. normal saline.

Results

Uptake of ^{125}I-C3 and ^{125}I-C5 by Platelets. As shown in Table I, activation of complement on the platelet surface by either the classic or the alternative pathway led to uptake of 40–60,000 molecules of C3 per cell and 3–4,000 molecules of C5. However, incubation of platelets with complement in the presence of thrombin led to a similar uptake of C5 but to a much reduced uptake of C3.

Incubation of complement with erythrocytes or leukocytes in the presence of thrombin led to an uptake of C3 and C5 that was not distinguishable from the EDTA-containing control.

Morphologic Demonstration of Thrombin-Induced Uptake of C3 and

TABLE I

Uptake of C3 and C5 by Platelets When Complement is Activated by Three Different Mechanisms

Activation of complement induced by*	Number of molecules per platelet	
	C3	C5
Antibody	39,800	4,200
Inulin	61,600	2,900
Thrombin	4,400	3,600

* For details of method of activation of complement, see text.

C5. Distribution of ferritin conjugated to anti-C3 or anti-C5 on the surface of the platelet membrane is shown in Fig. 1. When complement was activated by thrombin, the amount of bound labeled anti-C3 was minimal as compared to the uptake when complement was activated by either the classic (Fig. 1c) or alternative mechanism (not shown). The platelet membranes from the thrombin-mediated reaction demonstrated large areas totally devoid of ferritin, and the ferritin when present was found to be localized in large clusters of 20–30 molecules. The uptake and distribution of ferritin conjugated to anti-C5 (Fig. 1b) were similar to that seen when complement was activated on the platelet surface by the antibody-mediated classic mechanism (Fig. 1d). Fig. 2a and b are stained sections of platelets reacted with thrombin and complement reacted with ferritin-conjugated anti-C3 (Fig. 2a) or anti-C5 (Fig. 2b).

Mechanism of Activation of Complement by Thrombin. We have previously reported (6) that subsequent to the incubation of platelets, thrombin, and a source of complement, ultrastructural lesions can be visualized on the surface of the platelet. To elucidate the mechanism of activation of complement, platelets were incubated with thrombin in the presence of serum from a patient deficient in C2. Ultrastructural lesions were still seen on the platelets, indicating that this total blockage of the classic mechanism had not prevented formation of the lesions. A blockage of the alternate pathway was initiated by heating serum at 50°C for 30 min to inactivate Factor B. Platelets incubated in heated serum in the presence of thrombin still exhibited ultrastructural lesions.

The Effect of Aluminum Hydroxide-Absorbed Serum on Thrombin-Induced Aggregation of Washed Platelets. Fig. 3 demonstrates aggregation of 0.3-ml platelets by 0.1 U of thrombin either in the presence (a) or absence (b) of 10 μl of aluminum hydroxide-absorbed serum. The $Al(OH)_3$-absorbed serum in the absence of thrombin induced no platelet aggregation.

The Effect of $Al(OH)_3$-Absorbed Serum on Thrombin-Induced Release of Serotonin. Release of ^{14}C-serotonin from platelets induced by varying amounts of thrombin either in the presence or absence of aluminum hydroxide-absorbed serum is shown in Table II. There was a two- to fourfold increase in the ^{14}C-serotonin released by thrombin in the presence of the serum over that obtained with thrombin alone. This was most evident at the lower dose range of thrombin (0.025 and 0.05 U).

The Effect of $Al(OH)_3$-Absorbed Serum on Release of Lactic Dehydrogenase (LDH). Under the conditions utilized in the present study, namely 0.1 U

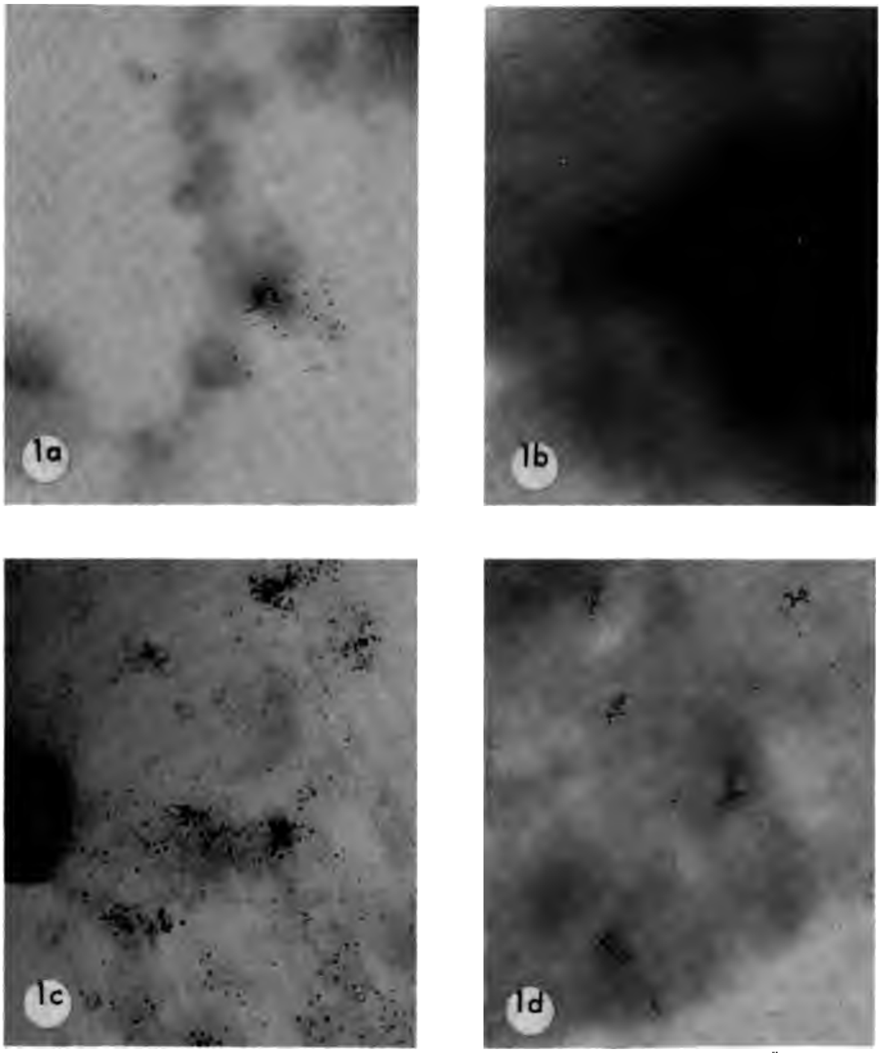

FIG. 1. Preparation of platelet membranes reacted with ferritin-conjugated antibody. Unstained. ×110,000. (a and b): Complement activated by thrombin. (a) Anti-C3 conjugated with ferritin; (b) anti-C5 conjugated with ferritin; (c and d) complement activated by antibody-mediated classic mechanism; (c) anti-C3 conjugated with ferritin; (d) anti-C5 conjugated with ferritin.

thrombin per 0.3-ml platelets, no LDH was liberated from platelets by thrombin either in the presence or absence of aluminum hydroxide-absorbed serum even after an incubation period of 2 h at 37°C.

The Effect of KSCN Treatment of Al(OH)$_3$-Absorbed Serum. Al(OH)$_3$-absorbed serum when further treated with KSCN under conditions known to inactivate C3, C4, and C5 (15) failed to enhance significantly the release of serotonin by thrombin (Fig. 4). However, addition of 25 μg of highly purified C3 and 3.1 μg of C5 restored the enhancing ability of the serum. Addition of 17.6 μg of C4 had little or no effect.

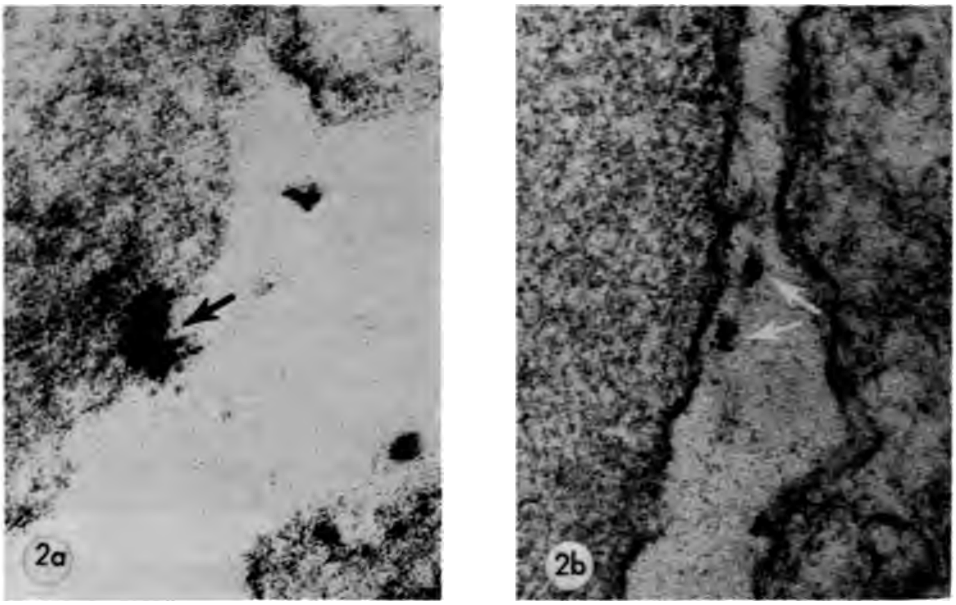

FIG. 2. Platelets reacted with thrombin and complement; then ferritin-conjugated antibody; sectioned and stained × 100,000. Arrows indicate clumps of ferritin molecules. (a) Anti-C3 conjugated with ferritin; (b) anti-C5 conjugated with ferritin.

Use of Purified Complement Components in Platelet Aggregation and Release Reactions. Figs. 4 and 5 show the results of multiple experiments. The large standard deviations are the result of a considerable variation in the degree of agglutination and release of serotonin given by platelet preparations from different donors. Whether the difference is due to drugs, food, etc. in different donors was not determined. However, with a single batch of platelets on a given day, the trends were always reproducible.

The sequence of addition of thrombin and purified complement components was important in determining the extent of platelet ^{14}C-serotonin release. When thrombin was added before C3–C9, serotonin release was enhanced compared to the experiments in which C3–C9 was added before thrombin (Fig. 5). In multiple experiments, the highest degree of serotonin release was obtained when C3, C5, C6, C7, C8, and C9 were added. However, some enhancement of release of serotonin was obtained when C3 alone was added or when C3 and C5 were added or when C3, C5, C6, and C7 were added. Some release enhancement was also noted in the presence of C8 and C9. Despite the large standard deviation, the enhancement of serotonin release by C3–C7 was always less than that produced by C3–C9 with any single batch of platelets.

As a result of these experiments, the question arose as to whether C8 and C9 were bound to the platelet surface and were being utilized in the release reaction. To answer this question, 0.3 ml platelets were incubated with 20 μl of the γ-globulin fraction of anti-C9 for 15 min at 37°C. After this period the tube was transferred to the platelet aggregometer and 0.1 U of thrombin was added, followed by 10 μl each of C3, C5, C6, and C7. It was found that the enhancement of aggregation and release initiated by C3–C7 was totally abolished by incuba-

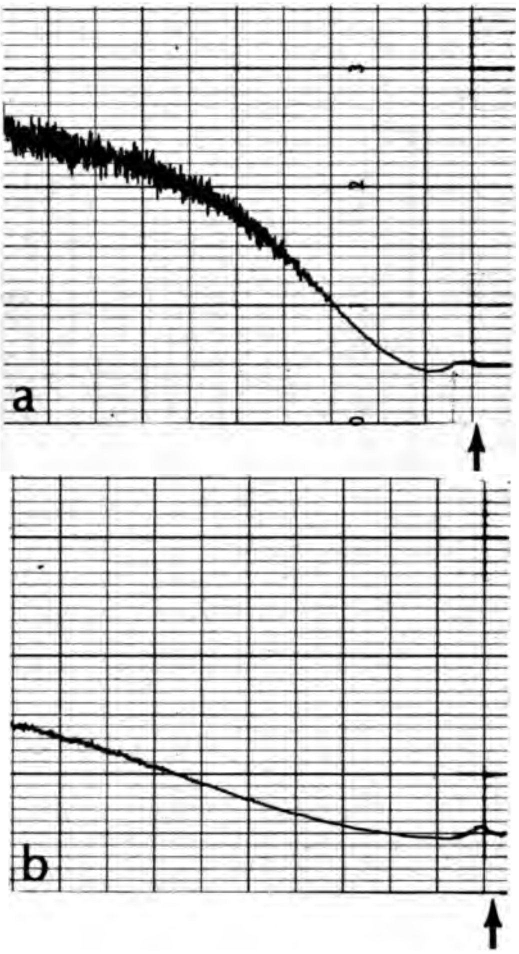

FIG. 3. Platelet aggregometer tracing of thrombin-mediated platelet aggregation: (a) 0.3 ml washed platelets in the presence of 10 μl of aluminum hydroxide-absorbed serum; (b) 0.1 U thrombin-0.3 ml washed platelets. Arrow indicates addition of thrombin.

TABLE II

Comparison of Thrombin-Induced Release of ^{14}C-Serotonin in the Presence and Absence of Complement

Units of thrombin*	Al(OH)$_3$ absorbed serum‡	Release of ^{14}C-serotonin
		%
0.025	–	0
0.05	–	26
0.1	–	59
0.025	+	28
0.05	+	64
0.1	+	65

* Added to 0.3 ml of washed platelets.

‡ 10 μl added.

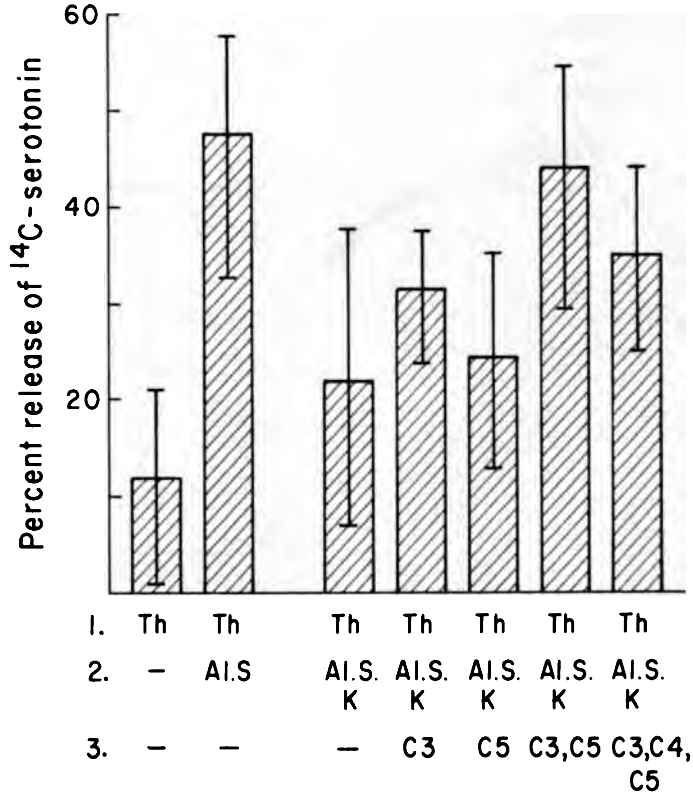

FIG. 4. Effect of various reagents on thrombin-induced release of platelet serotonin. Th, thrombin; Al.S, fresh human serum absorbed with aluminum hydroxide; Al.S.K., fresh human serum absorbed with aluminum hydroxide then treated with potassium thiocyanate (see text). 1 and 2 indicate sequence of addition.

tion of the platelets with anti-C9. When anti-C9 was replaced by anti-human serum albumin, there was no effect on subsequent platelet reactivity. The enhancement of aggregation of release by C8 and C9 alone was similarly inhibited by antisera to either C3 or to C5 (Fig. 6).

Thus C3, C5, C6, C7, C8, and C9, when mixed together in the absence of any known activating components of the classic or alternative mechanism, produced an enhancement of thrombin-induced aggregation and release equivalent to that induced by $Al(OH)_3$ serum.

Mechanism of Activation of Complement by Thrombin. The observation that addition of C3, C5, C6, C7, C8, and C9 in the absence of any known activator of complement led to enhanced thrombin-induced aggregation and release of serotonin, suggested the possibility that complement was activated by a method distinct from the known classic or alternative mechanisms. To clarify this possibility, serum reagents were utilized in which a key component of either system was absent. The serum totally deficient in C2 was utilized as a serum in which the classic mechanism was inhibited. As shown in Fig. 7, when this serum was absorbed in the usual way with aluminum hydroxide and utilized as a source of complement, thrombin-induced release of serotonin was

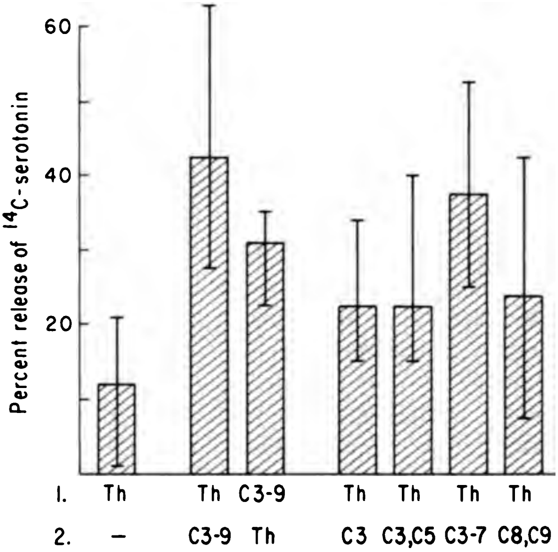

FIG. 5. Effect of various reagents and purified complement components on thrombin-induced release of platelet serotonin. C3–C7: complement components C3, C5, C6, C7. C3–C9: complement components C3, C5, C6, C7, C8, and C9. Other abbreviations are the same as in the legend to Fig. 4

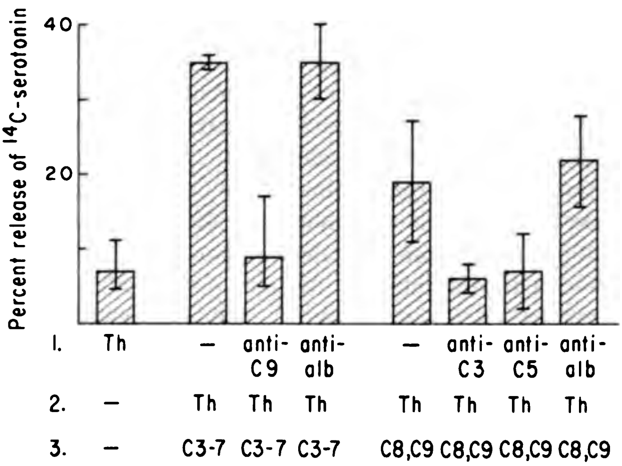

FIG. 6. The effect of antibody on thrombin-induced release of platelet serotonin, 1, 2, and 3 indicate sequence of addition. Anti-alb: anti-human serum albumin. For methodology, see text.

equal to that obtained with C2-sufficient normal serum. A blockage of the alternative mechanism was produced by heat inactivation (50°C, 30 min) of Factor B. Aluminum hydroxide-absorbed heated serum was indistinguishable from unheated serum in mediating release of serotonin. Further, blockage of both the classic and alternative mechanisms was produced by heating at 50°C for 30 min the C2-deficient serum that had been absorbed with aluminum hydroxide. This serum reagent was also indistinguishable from normal serum

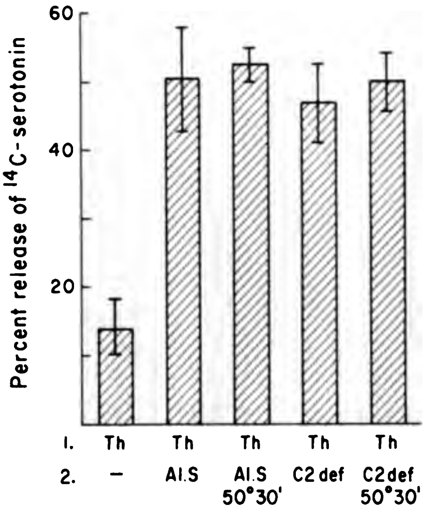

FIG. 7. Effect of various reagents on thrombin-induced release of platelet serotonin. C2 def, serum from patient deficient in C2 absorbed with aluminum hydroxide. C2 def 50°C30', serum from patient deficient in C2 absorbed with aluminum hydroxide, then heated at 50°C for 30 min. Other abbreviations are the same as in the legend to Fig. 4.

in inducing enhancement of thrombin-induced release of serotonin. Platelet aggregation in each experiment corresponded to serotonin release.

To ascertain whether platelet-bound C2 or Factor B were contributing to the release reaction in the absence of exogenous C2 or Factor B, experiments were performed in which the platelets were reacted with antibody to either C2 or Factor B before they were exposed to C2-deficient serum, Factor B-depleted serum, or C2-deficient serum that had been heated. Neither antibody caused any decrease in the platelet aggregation or release of serotonin.

DFP Treatment of Thrombin. Treatment of thrombin with DFP totally inhibited its ability to aggregate platelets and to release serotonin both in the presence and absence of complement.

Discussion

In a previous communication (6), we demonstrated the presence of ultrastructural lesions on the surface of platelets that had been incubated with thrombin and complement. We showed that these morphologic membrane perturbations were dependent on the presence of thrombin and C3. In the present study we have extended these studies and demonstrated cellular uptake of C3 and C5 by the platelets and have investigated the role of complement in thrombin-mediated platelet functions.

Utilizing radioactively labeled C3 and C5, we were able to demonstrate both C3 and C5 on the platelet surface. The uptake of C3 was very much less than the uptake seen when complement was activated on the platelet surface by either the classic mechanism or the alternative mechanism. However, the uptake of C5 was similar to that produced by activation of the classic mechanism. That the platelet membrane was an integral component of this reaction was demonstrated by the finding that a similar activation of complement and uptake of C3 and C5 was not seen when the platelets were replaced with either erythrocytes or leukocytes.

The uptake and spatial organization of these components were visualized utilizing ferritin conjugated to anti-C3 or anti-C5 and electron microscopy. Whereas the uptake of ferritin conjugated to anti-C5 was similar to that found when complement was activated by the classic mechanism, the uptake of ferritin conjugated to anti-C3 was totally different from that seen when complement was activated by either the classic or the alternative mechanism. When complement was activated by the antibody-mediated classic mechanism, the distribution of the C3 ferritin marker was similar to that published (24) for its distribution on erythrocytes. However, after thrombin-mediated complement activity, there were very few molecules of C3 per cell. In fact, the number of C3 molecules approximated the number of C5 molecules, whereas when complement was activated by the classic mechanism, a 10-fold greater number of C3 molecules was obtained than of C5 molecules, and by the alternative mechanism a 20-fold difference was seen. When the molecules were visualized on the platelet surface utilizing a ferritin marker, it was found that large areas of the platelet membrane had no C3 and the C3 that was present was focalized in large clusters visualized as 20–30 ferritin molecules.

Zimmerman and Kolb (4) have reported uptake of C3 and the C5–C9 complex when platelets are incubated in serum. However, it is not clear whether the mechanism of uptake is the same as that demonstrated in the present study.

The results of our studies led us to investigate the role of complement in thrombin-mediated platelet function. Initially in these studies we utilized aluminum hydroxide-absorbed serum as a source of complement. With this reagent we found increased platelet aggregation and release of serotonin in the presence of complement. That the release of serotonin was a nonlytic process was demonstrated by the finding that no lactic dehydrogenase was liberated during this reaction. A role for complement in thrombin-mediated platelet function has been previously suggested (5). These authors demonstrated that retraction and lysis of thrombin-induced blood clots were found to be inhibited by monospecific antisera to C3 and C4. Also thrombin was shown to cleave C3 into C3a-like and C3b-like peptides. However, the C3a produced by cleavage of C3 with thrombin had no anaphylatoxin activity (14).

The use of purified components of complement in place of aluminum hydroxide-absorbed serum as a source of complement demonstrated that maximum thrombin-induced platelet aggregation and release could be achieved by the addition of C3, C5, C6, C7, C8, and C9 in the absence of any previously described C3 convertase. Further, the sequence of addition of thrombin and complement was important. Considerably more serotonin was released when thrombin was added before the components of complement than when the components were added before the thrombin. All six components were required for maximal aggregation and release. However, C3, C5, C6, and C7 induced a considerable enhancement over that obtained with thrombin in the absence of complement. The release induced by C3–C7 was facilitated by platelet-bound C9 (and probably C8) as demonstrated by the fact that the C3-C7 reactivity was inhibited if the platelets were first treated with anti-C9 (Fig. 6). Some enhancement of release was obtained when C8 and C9 alone were added. That this release was

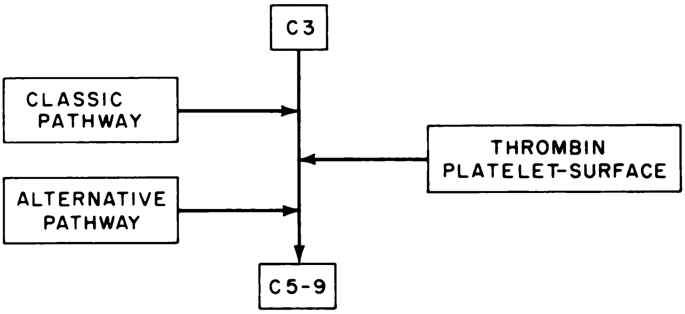

FIG. 8. Complement activation mechanisms. A new third pathway.

due to the presence of platelet-bound C3 and C5 was evidenced by the finding that it was inhibited by anti-C3 and anti-C5.

Thus the data suggest that thrombin and the platelet membrane generate a C3 convertase that is distinct from those previously described as a consequence of the activation of the classic or alternative mechanism. Because the sequence of addition of thrombin and C3–C9 is important, it appears that if thrombin is allowed first to interact with the platelet (presumably the thrombin-receptor; 25–27), this interaction brings the activation of the C3–C9 onto the platelet surface in contrast to the reaction taking place in the fluid phase. It will be of some interest in the future to determine whether the membrane attack complex (C5b–C9) is assembled on the platelet surface as a consequence of thrombin action.

Confirmatory data for lack of activation of either the classic mechanism or the alternative mechanism were obtained in experiments in which C2-deficient or Factor B-depleted serum was used. These sera, used as a source of complement, induced enhancement of thrombin-mediated aggregation and release of serotonin which was indistinguishable from that obtained with normal serum. Cell-bound C2 or Factor B was not responsible for this reactivity because prior treatment of the platelets with the corresponding antibody had no effect on the reactivity of the sera. We suggest, as illustrated in Fig. 8, that there exists a third mechanism leading to the activation of complement. This pathway requires thrombin and the platelet membrane and enters the known complement sequence at the C3 stage. It will be of great interest to determine whether other platelet membrane perturbants, such as collagen or ADP, have similar properties. Complement is not essential for thrombin-mediated platelet function; however, considerable enhancement of its reactivity can be obtained by the addition of C3, C5, C6, C7, C8, and C9.

Summary

Thrombin-mediated platelet membrane-specific uptake of C3 and C5 was demonstrated by radiolabeled components and was visualized electron microscopically utilizing a ferritin marker conjugated to monospecific antibody to each component. The role of complement in thrombin-induced platelet function was determined. Though complement was not essential for thrombin-induced platelet aggregation and release of serotonin, these activities were significantly

increased if complement was present. The release of serotonin was found to be a nonlytic process because under the conditions employed, no lactic dehydrogenase was released. The activation of complement was induced by a mechanism which has not been previously described. Thrombin associated with the platelet membrane presumably formed a C3 convertase that entered the known complement sequence at the C3 stage and proceeded to activate the terminal components through the known sequence to C9.

The authors gratefully acknowledge the skillful technical assistance of Ms. Eileen Cotty, Ms. Bonnye Benjamin, and Ms. Linda Metakis.

Received for publication 17 January 1978.

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THE GENERATION AND REGULATION OF LYMPHOCYTE POPULATIONS

Evidence from Differentiative Induction Systems in Vitro*

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As the cells that are programmed to generate the various sets of immunocompetent lymphocytes progress along their several pathways they pass through discrete phases of differentiation that can be recognized by the selective expression of cell surface components. Detailed investigation of the nature and mechanisms of these differentiative steps has been much facilitated by the development of short-term assays in which some of these phenotypes are induced in vitro (1–4). We present here an account of our findings with induction assays that monitor an early step in T-cell differentiation, marked by acquisition of the surface component Thy-1, and two steps in B-cell differentiation, the first marked by acquisition of complement receptors (CR)[1] (5) and the second by acquisition of the surface component PC (plasma cell antigen) (6).

Materials and Methods

Congenic α-Thy-1.2 Antiserum [A-Thy-1.1 × AKR-H-2^b anti-A strain leukemia ASL1]. For use in induction assays this antiserum was preabsorbed with Thy-1.1 thymocytes and leukemia cells until negative in cytotoxicity assays with congenic Thy-1.1 thymocytes. Resultant specific titers against congenic Thy-1.2 thymocytes were >1:2,000. Without this preabsorption, unidentified contaminant antibody (possibly murine leukemia virus-related) can give erroneous positive results. Anti-Thy-1.2 antiserum of lower specific titer is inadequate for induction assays. (The same requirements for preabsorption and high specific titer apply equally to the alternative antiserum α-TL, which has given results similar to α-Thy-1 in all respects [7].)

PC Antisera and PC Induction Assay. The same considerations of titer and specificity apply to α-PC serum (DBA/2 × B6)F_1 anti-BALB/c myeloma MOPC-70A (6), in PC induction assays. This antiserum was preabsorbed with thymus and spleen of PC^- strains of mice, including B6, the resulting specific cytotoxicity titer being 1:64 against MOPC-70A cells with guinea pig serum (GPS) as the source of complement), and 1:32 or 1:64 against induced Peyer's patch cells, with rabbit C. We found Peyer's patches to be the richest source of PC-inducible Ig^+:CR^+:PC^- cells, but their proportion is still too low for a satisfactory induction assay. Therefore we used Peyer's patch

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* Supported in part by grants HD-08415, CA-22241, CA-16889, and AI-13374 from the National Institutes of Health.

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[1] *Abbreviations used in this paper*: α, anti; B6, C57BL/6; BSA, bovine serum albumin, cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; C, complement; CR, complement receptor; EAC, erythrocyte antibody complement complex; EDTA, ethylene diamine tetraacetate; FcR, Fc receptor; GPS, guinea pig serum; MuLV, murine leukemia virus; PC, plasma cell antigen; PDE, phosphodiesterase; PGE_1, prostaglandin E_1; Poly A:U, polyadenylic-polyuridylic acid; R-α-MIg, rabbit anti-mouse immunoglobulin; SRBC, sheep erythrocytes.

cells from mice stimulated with *Corynebacterium parvum* (1 mg per animal, i.p., 5–7 days before harvest), cleared of dead cells by a two-step bovine serum albumin (BSA) gradient. This yielded 25–30% PC-inducible cells (scored with preabsorbed α-PC serum diluted 1:16, after 2.5–3 h incubation).

Other Antisera. Rabbit anti-mouse immunoglobulin (R-α-MIg) consisted of immunoabsorbent-purified α-MIgM combined with immunoabsorbent-purified α-F(ab)$_2$ of MIgG (4), used in cytotoxicity procedures at a final concentration of 0.1 mg/ml. Purified 19S fraction of rabbit α-sheep erythrocytes (SRBC) (Cordis Laboratories, Inc., Miami, Fla.) diluted 1:100 was used in the CR assay.

Complement (C). Ample rabbit C is crucial to cytoxicity assays for induction of cell-surface markers. GPS is generally inadequate. Rabbits were screened for low natural cytotoxicity for thymocytes and high C activity in a system (α-Lyt-2.2) which demands a high level of C. For each pool of C, usually one or two rabbits were selected from 20–30 pretested rabbits.

Thymopoietin (8) and ubiquitin (3) were lyophilized in 1-μg lots, with 100 μg BSA to reduce loss from adsorption to glass, and dissolved in RMPI-1640 immediately before use.

Pharmacological Agents. cAMP (adenosine 3′:5′ monophosphoric acid); DBcAMP (N_6O_2-dibutyryl-adenosine 3′:5′ monophosphoric acid); theophylline; DL-isoproterenol; L-isoproterenol; DL-propranolol; epinephrine (L-epinephrine); imidazole, carbachol (carbamylcholine chloride); cycloheximide (Sigma Chemical Co., St. Louis, Mo.). We thank Dr. M. Weksler (Cornell Medical College) for providing L-propranolol, Dr. J. Pike (Upjohn Co., Kalamazoo, Mich.) for PGE$_1$ (prostaglandin E$_1$), Dr. C. Henney (Johns Hopkins University) for choleratoxin (cholera-enterotoxin) and 8-bromo-cGMP (8-bromo-cyclic guanosine monophosphate), Dr. G. Renoux (Tours-Cedex, France) for poly A:U (polyadenylic-polyuridylic acid), and Dr. C. Galanos (Freiburg) for lipopolysaccharide (endotoxin) and lipid A.

Erythrocyte Antibody Complement Complex (EAC) Indicator Cells. SRBC were washed and suspended in medium 199 (5%); an equal volume of rabbit α-SRBC added; incubated (37°C, 15 min); washed three times and resuspended (5%); added to an equal volume of normal AKR mouse serum (diluted 1:5); further incubated (37°C, 45 min); washed twice, and resuspended in RPMI-1640 (5%).

Density-Gradient Centrifugation to Fractionate the Spleen Cell Population for Enrichment of Inducible Cells (illustrated in Fig. 1). 5–10 × 10^7 washed spleen cells (85–95% viable) were pelleted in a 5-ml Beckman cellulose nitrate tube (150 *g*). The supernate was removed, and the walls of the tube dried. The cells were suspended in 1 ml 35% BSA (Pathocyte 5, lot 21, 22; Miles Laboratories Inc., Miles Research Products, Elkhart, Ind.), overlaid with four 1-ml layers of BSA (29:26:23:10%) as shown in Fig. 1, and the gradient was centrifuged (Beckman model L; SW50.1 rotor; 13,000 rpm; 30 min). Each layer was aspirated in turn through each interface to the midpoint of the next layer, diluted at least 1:15 with RPMI-1640, and the cells washed twice before use in induction assays. Some lots of BSA were unsuitable because they contained >1 μg/ml endotoxin, itself an inducer (9), and Ficoll proved unsatisfactory because some lots had inducing activity.

The Standard Cell Population for Common use in Thy-1 and CR Induction Assays. The standard cell population used as the common source of cells for prothymocyte and pro-CR$^+$ cell assays was the combined B and C layers indicated by the arrows in Fig. 1. To avoid high backgrounds of pre-existing CR$^+$ cells in the CR induction assay, we used spleen from *nu/nu* mice, which if healthy have fewer CR$^+$ cells than *nu/+* and other phenotypically normal mice (Dr. M. C. Gelfand, personal communication; and M. P. Scheid, unpublished observations). We used *nu/nu* mice of our own colony (bred on a BALB/c background and maintained under conventional conditions), aged 1–2 mo. Spleen cells of normal mice, depleted of CR$^+$ cells by sedimentation as rosettes with EAC (5), were used with equal success for preparing the standard cell population when *nu/nu* mice were not available.

General Description of the Induction Assays. The cells (1–5 × 10^{-7} cells/ml RPMI-1640) were distributed into 5-ml plastic tissue culture tubes (BioQuest, BBL & Falcon Products, Becton, Dickinson & Co., Cockeysville, Md.). An equal volume of inducer (in RPMI-1640) was added. After incubation for 10 min at 37°C in a waterbath (to equilibriate temperature) the tubes were placed in a humidified 5% CO$_2$ incubator at 37°C for 2–2.5 h. Afterwards the cells were washed twice in RPMI-1640, resuspended in medium 199 for cytotoxicity assays, or RPMI-1640 for CR assays.

The CR^+ phenotype for assay of B cells (5) was scored as follows: 0.3 ml EAC (0.5% suspension) was added to 0.3 ml of the induced cell suspension (2×10^6/ml), and EDTA to 0.01 M, in a Beem plastic electron-microscopy capsule (Ernest F. Fullam, Inc., Schenectady, N.Y.). The suspension was shaken at 37°C in a waterbath and then placed on ice; (the capsules can be held at 4°C overnight without change in the readings). A rosette was defined as a cell binding ≥ 3 EAC. At least 300 cells from each tube were counted, and the proportion of rosetted cells expressed as the percentage of total nucleated cells (rosetted plus nonrosetted). Controls for which due allowance was made in calculating CR-specific complex rosetting and induction indices included: (*a*) erythrocyte antibody substituted for EAC, and (*b*) omission of the cells tested for induction.

Controls for Background Induction and for Loss of Surface Markers during Induction Assays. Each assay was controlled for the following three factors: (*a*) marker-positive cells in the starting population: this was estimated by cytotoxicity assays on the standard cell population immediately before induction assay (Control A). (*b*) Induction by contaminants: contaminants such as endotoxin in some lots of BSA, encountered during fractionation of spleen cells, produce a background of induced cells after 2 h of culture. This was estimated by control cultures to which no inducer was added (Control B). Control B should equal Control A if there is no background induction. (*c*) Loss of surface markers: the proportion of cells that register FcR, Ig, or CR, but never Thy-1 or PC, may fall in controls at 37°C without added inducer (Control B). This does not occur at 0°C. Hence Control C (for induction assays of FcR, Ig, and CR) which is the same as Control B but at 0°C. Any test in which controls A, B, and C differed significantly was discarded.

Calculation of Percentages of Cells Induced. The formula for all three markers (Thy-1, CR, PC) was $(a - b)/(100 - b) \times 100$, where a = percent marker-positive cells in the presence of inducer, and b = percent marker-positive cells in the absence of inducer. The values of b in a series of 12 representative tests were 8 (SEM ± 5) for the prothymocyte assay, 13 (SEM ± 5) for the pro-CR^+ assay, and generally <5 for the PC assay.

Elimination of Thy-1$^+$ and Ig$^+$ Cell Populations. Washed spleen cells were suspended (2×10^7/ml) in α-Thy-1.2 (1:50) or α-MIg (1:100); incubated at 4°C for 30 min; washed once; suspended at the original concentration in rabbit serum (C) diluted 1:15; incubated (shaken) for 30 min at 37°C in a waterbath; counted in trypan blue; layered on a two-step (10–35%) BSA gradient; and centrifuged to separate the dead cells. Cytotoxicity assays of the viable cells recovered at the interface showed >90% to be Thy-1$^-$ or Ig$^-$, respectively. A control in which antiserum was omitted was always run, to confirm that the pool of C in use still gave negligible cytolysis.

Results

I. *Enrichment of Precursor Cells by Density Gradient Centrifugation; Distribution of Cell Types in the Standard Gradients.* The term prothymocytes (10) is used for the cells in bone marrow and spleen which lack the T-cell surface antigens Thy 1:TL:Lyt but express them when induced to differentiate in vitro (1).

The term pro-CR^+ cells refers to cells of the B-lymphocyte lineage, in the spleen and to a lesser extent in bone marrow, which do not express CR but do so when induced to differentiate in vitro (granulocytes and macrophages are CR^+, but form CR rosettes only in the presence of divalent cations [11] which EDTA excludes in our CR assay).

Fig. 1 shows the BSA gradient used and (for A strain spleen) the distribution profiles of: (*a*) prothymocytes, (*b*) pre-existing Thy-1$^+$ cells, (*c*) pro-CR^+ cells, and (*d*) pre-existing CR^+ cells. The profiles of all four cell types, shown on the right in Fig. 1, are different from one another, implying that prothymocytes and pro-CR^+ cells are different inducible populations.

II. *The Kinetics of T-Cell and CR^+ B-Cell Induction.* Demonstrable induction of Thy-1 and TL expression requires 2 h (1). In a preparatory study we found induction of CR^+ cells by ubiquitin to be virtually maximal at 2 h in cells

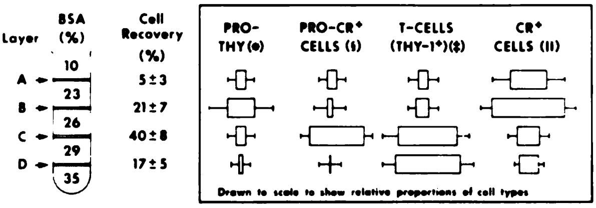

FIG. 1. Distribution of spleen cell types in the standard BSA gradient.

* Prothymocytes: phenotype TL^-:Thy 1^-, inducible to express all thymocyte surface markers; § Pro-CR^+ cells: B-lineage cells inducible to express CR; phenotype Ig^+:Ia^+:CR^-; ‡ T cells: phenotype TL^-:Thy 1^+:Lyt^+. ||CR^+ cells: Ig^+:Ia^+:CR^+. Layers B and C combined, generally from nu/nu mice, are referred to as the standard cell population for induction (see Materials and Methods). The data shown here are averages for 5-10 × 10^7 spleen cells, from A strain mice aged 2-3 mo, on the standard gradient (volume of each of the five layers = 1 ml).

from mice aged 2-3 mo; a longer induction period may be required for cells from younger donors.

Induction does not require presence of inducer throughout the 2-h period. 10 min, the shortest time tested, sufficed for near-maximal registration of induction (at 2 h) by ubiquitin in both assays and by thymopoietin selectively in the T-cell assay. Thus the time required for manifestation of the induced phenotype is much longer than the time required for initiation by either inducer. These data suggest initiation by a surface ligand which triggers intermediary events leading to phenotypic expression. Mediation by cAMP is suggested by reports that its addition causes induction and that pharmacological interference with cAMP metabolism blocks induction, in both assays (2). We tested this further by adding imidazole during induction, in a concentration at which imidazole's known action is to decrease cellular cAMP and increase cGMP, probably by activating cellular cAMP phosphodiesterase (12). Imidazole inhibited induction in both assays completely when added 10 min after the inducer, and partially when added up to 30 min later (Table I). Thus the critical period for cAMP action appears to be the first 30 min of the induction process.

III. *Metabolic Requirement for Induction.* Prothymocyte induction requires cell metabolism; it is prevented by lowering the temperature and by inhibitors of transcription and translation (13). The same is true of pro-CR^+ B-cell induction; we find no CR induction at 4°C. In both assays, cycloheximide blocked induction completely at concentrations of 2.5 and 10 μg/ml (which reduce cellular incorporation of [^{3}H]leucine by about 95% [13]), and partially at 0.1 μg/ml (25% inhibition of [^{3}H]leucine uptake [13]).

IV. *Further Evidence that Prothymocytes and Pro-CR^+ Cells are Discrete Populations.* Fig. 2 shows: (*a*) that thymopoietin, throughout its entire range of effective concentration, generates only thymocytes (panels 1 and 2) whereas ubiquitin generates both thymocytes and CR^+ cells (panels 3 and 4). (*b*) Induction by ubiquitin is prevented by propranolol (10^{-5} M), a β-adrenergic blocking agent (14), in both assays (panels 3 and 4), whereas induction of prothymocytes by thymopoietin is not prevented by propranolol (panel 1).

If there were a single uniform immediate precursor population capable of differentiating to thymocytes or to CR^+ B cells, then induction of the population

TABLE I

Suppression of Prothymocyte and Pro-CR$^+$ B-Cell Induction by Imidazole

Ubiquitin (0.1 µg/ml)	Imidazole (10^{-7} M) added after:					Cells induced %			
	0	10	30 min	60	90	Pro-thymocyte assay		Pro-CR$^+$ cell assay	
						1st Exp.	2nd Exp.	1st Exp.	2nd Exp.
None						0 (Standard)	0	0 (Standard)	0
Present						15	20	16	22
None	+					−1	−4	−3	−3
Present	+					−8*	−5	−3	−4
Present		+				−6	−2	2	−1
Present			+			11	15	6	17
Present				+		16	24	10	15
Present					+	10	19	15	17

* High negative indices presumably reflect imidazole's suppression of the usual low background of cells induced in controls without an added inducer.

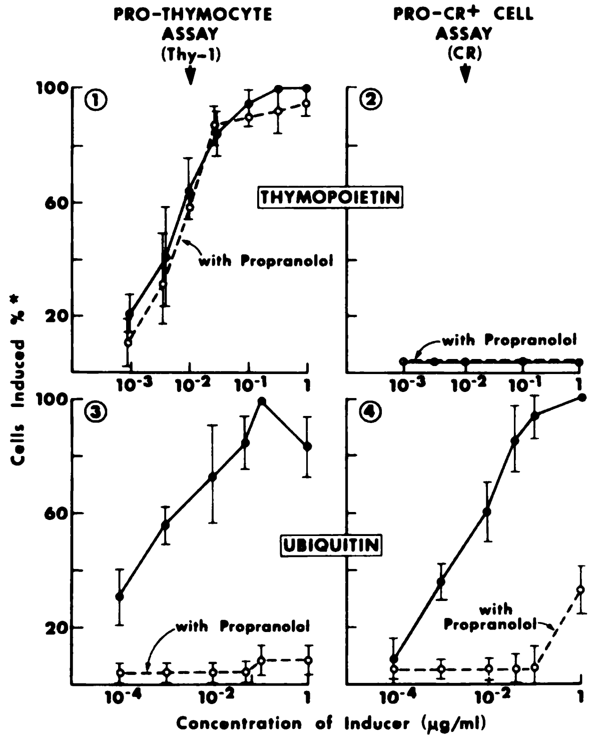

FIG. 2. Effects of propranolol (10^{-5} M) on induction by thymopoietin (panels 1 and 2) and ubiquitin (panels 3 and 4) in dual induction assays with the standard cell population. (Mean values for four separate sets.)

* Because the proportion of cells induced varies somewhat according to the cell preparations used in different experiments, the data shown are standardized as percent of maximal induction in the positive control (no inhibitor; optimal concentration of inducer) in each test.

TABLE II
Inductive Selectivity of Thymopoietin and of Ubiquitin, as Indicated by Sequential Induction Assays

First induction			Second induction		
Inducer (0.1 μg/ml)	Cells induced %		Inducer (0.1 μg/ml)	Cells induced %*	
	Pro-thymocyte assay	Pro-CR$^+$ cells assay		Pro-thymocyte assay	Pro-CR$^+$ cell assay
Thymopoietin	15 ± 7	<2	Ubiquitin	<2	40 ± 4
Ubiquitin	16 ± 5	23 ± 3	Ubiquitin	<2	35 ± 5
None	0 (Standard)	0 (Standard)	Ubiquitin	12 ± 3	25 ± 5

* Mean ± SEM for four experiments.

TABLE III
Prothymocyte Induction Assays with R-α-MIg-Depleted Cell Populations

Step 1: Cytolysis	Step 2: Induction Inducer:	Cells induced %*	
		Pro-thymocyte assay	Pro-CR$^+$ cell assay
	None	0 (Standard)	0 (Standard)
R-α-MIg + C	Thymopoietin (0.5 μg/ml)	26 ± 3	5 ± 3
	Endotoxin (30 μg/ml)	21 ± 4	−1 ± 5
	None	0 (Standard)	0 (Standard)
C Alone (control)	Thymopoietin (0.5 μg/ml)	22 ± 3	−3 ± 2
	Endotoxin (30 μg/ml)	19 ± 6	20 ± 5

The starting population for step 1 was unfractionated spleen cells, after which the standard cell population was prepared as usual.

* Mean ± SEM for four experiments.

by thymopoietin, which produces only thymocytes, should leave no cells capable of subsequently being induced to form CR$^+$ cells.

Table II shows data for experiments in which the standard cell population was treated with thymopoietin or with ubiquitin (first induction) and then with α-Thy 1 + C to eliminate the thymocytes induced. The cells were then subjected to a second induction, with ubiquitin. There was no further yield of T cells. The higher proportion of CR$^+$ cells after the second induction is due to the relative enrichment of CR$^+$ cells caused by elimination of the T cells after the first induction. This result is not compatible with generation of thymocytes and CR$^+$ cells from the same immediate precursor pool.

Table III shows data for experiments in which pro-CR$^+$ cells, which are Ig$^+$, were eliminated from the standard cell population by cytolysis with R-α-MIg + C. Subsequent induction revealed no loss of inducible prothymocytes. These data further support the conclusion that prothymocytes and pro-CR$^+$ B cells are distinct populations.

V. *Evidence That There are Different Receptors for Thymopoietin and for*

TABLE IV

Receptor Specificity: Effect of Excess Ubiquitin on Sensitivity to Induction by Ubiquitin and by Thymopoietin

Inducer*	Cells induced %‡	
	Pro-thymocyte assay	Pro-CR$^+$ cell assay
Thymopoietin	18 ± 5	1 ± 3
Ubiquitin	1 ± 3	1 ± 2
Thymopoietin + Ubiquitin	15 ± 7	1 ± 4
DBcAMP (1 mM) + Ubiquitin	13 ± 5	10 ± 2

* In a concentration of 0.5 μg/ml for thymopoietin, and 200 μg/ml (predetermined induction-inhibitory concentration) for ubiquitin.

‡ Mean ± SEM of three experiments.

Ubiquitin. The observations that thymopoietin induces only prothymocytes whereas ubiquitin also induces pro-CR$^+$ B cells, and that propranolol, which prevents binding of β-adrenergic agonists (14), blocks induction by ubiquitin but not by thymopoietin (Fig. 2), suggest that the two inducers react with different receptors. Furthermore, high concentrations of ubiquitin do not induce prothymocytes or pro-CR$^+$ cells (Table IV, line 2), but this block does not hinder induction of prothymocytes by thymopoietin (line 3), nor of prothymocytes or pro-CR$^+$ cells by DBcAMP (line 4).

VI. *Pharmacology of Induction.* The evidence above points to two distinct precursor populations which are precommitted to the thymocyte and B-cell phenotypes, respectively, and to a common intermediary mechanism, probably involving cAMP. Pharmacological evidence supports this interpretation.

CYCLIC NUCLEOTIDES. Because cAMP and DBcAMP are inducers (2), cGMP should have counter-effects if lymphocyte induction conforms to known cAMP-mediated systems (14). Panels 1 and 2 in Fig. 3 give dose-response curves for DBcAMP and show no induction by cGMP (8-bromo cGMP) in either assay over the same entire range of concentrations. But cGMP prevented induction by ubiquitin in both assays when added 10 min beforehand (panels 3 and 4), and so did carbachol (panels 3 and 4), an acetylcholine analog which raises cellular levels of cGMP in lymphocytes (16, 17).

PHARMACOLOGICAL AGENTS AFFECTING CYCLIC NUCLEOTIDE PHOSPHODIESTERASE (PDE) ACTIVITY. Imidazole activates PDE and lowers the ratio of cAMP to cGMP in lymphocytes (12). We tested theophylline and imidazole with optimal and suboptimal concentrations of both thymopoietin and ubiquitin. Theophylline (which at the concentration used, 2 × 10^{-3} M, had no measurable inductive effect) enhanced the activity of suboptimal concentrations of both thymopoietin and ubiquitin, but suppressed induction by optimal concentrations of both inducers. This suppression is predictable from the dose-response curves for cAMP (Fig. 3, panels 1 and 2) which show inhibition of its own inductive action at high concentration. We also found that inclusion of cAMP with optimal and suboptimal concentrations of either thymopoietin or ubiquitin reproduced these effects of theophylline. In contrast to theophylline, which augments induction, imidazole (10^{-7} M) suppressed induction over the entire response range of

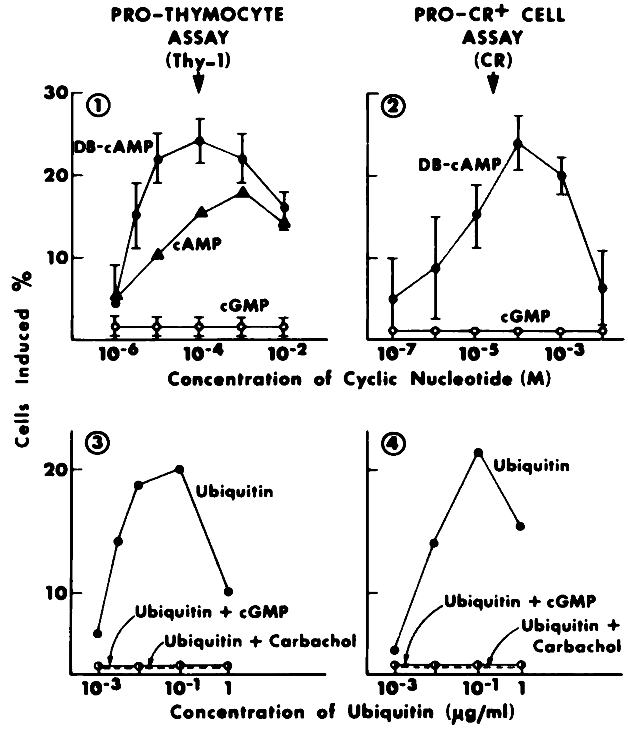

FIG. 3. Cyclic nucleotides. The dose-response curves for induction by DBcAMP and cAMP (panels 1 and 2) show inhibition at high concentrations. There is no induction by cGMP (8-bromo-cGMP) at any concentration (panels 1 and 2), but cGMP (10^{-3} M), and carbachol (10^{-8} M), inhibit induction by ubiquitin if added 10 min beforehand (panels 3 and 4). (Mean values of four separate tests.)

effective concentrations of thymopoietin or ubiquitin. (In higher concentrations, imidazole itself is an inducer, probably for reasons other than its influence on cAMP PDE [12].)

PHARMACOLOGICAL AGENTS ACTIVATING ADENYLATE CYCLASE: CHOLERATOXIN AND ADRENERGIC COMPOUNDS (Fig. 4). Choleratoxin and β-adrenergic agents have been characterized pharmacologically as effective stimulators of adenylate cyclase in nearly every tissue tested, including lymphocytes (14, 15, 17, 20). Both are inducers in both our assays. Choleratoxin gave maximal induction of Thy-1$^+$ cells at a concentration of 1 μg/ml (panel 1) and of CR$^+$ cells at 0.01 μg/ml (panel 2). In both assays induction by choleratoxin was prevented by imidazole (10^{-7} M) added 10 min before the inducer, whereas theophylline (10^{-3} M) enhanced induction by suboptimal concentrations of choleratoxin.

The adrenergic agent epinephrine (panels 3 and 4) gave maximal induction of both Thy-1$^+$ and CR$^+$ cells at a concentration of 10^{-4} M. Both inductions were inhibited by incubation (for 10 min) with propranolol (10^{-5} M) before the addition of epinephrine; whereas in both assays phentolamine (an α-adrenergic blocking agent), at the same concentration and under the same conditions, enhanced induction by suboptimal concentrations of epinephrine, and reduced induction by optimal concentrations of epinephrine. This suggests that the β-receptor, but not the α-receptor, serves for induction in both assays, and that

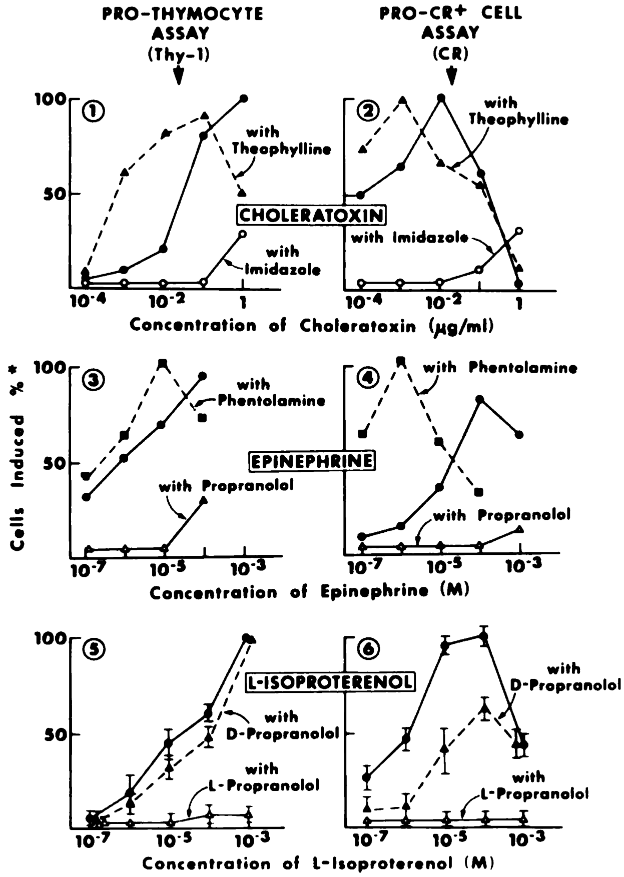

FIG. 4. Induction by three activators of adenylate cyclase: choleratoxin, epinephrine, and L-isoproterenol. The pharmacological data shown conform to the interpretation that activation of adenylate cyclase leads to induction regardless of which adenylate-cyclase-linked receptor is engaged. Thus induction by choleratoxin was inhibited by imidazole (10^{-7} M) and enhanced by theophylline (10^{-3} M) (panels 1 and 2). Induction by epinephrine was inhibited by propranolol (10^{-5} M) and enhanced by phentolamine (10^{-5} M) (panels 3 and 4). Induction of L-isoproterenol was inhibited by L-propranolol (10^{-5} M) rather than by D-propranolol (10^{-5} M) (panels 5 and 6). *Data standardized as percent maximal induction (see footnote to Fig. 2). (Mean values for three to four tests.)

effects of epinephrine mediated by α-adrenergic receptors, known in other systems (18), antagonize induction.

L-isoproterenol, a selective β-adrenergic agonist (18, 19) was inductive in both assays (panels 5 and 6). L-propranolol (10^{-5} M) completely inhibited induction by L-isoproterenol, in concentrations up to 10^{-3} M, in both assays. By contrast, D-propranolol (10^{-5} M), the dextrorotatory d-(+) isomer, did not significantly affect prothymocyte induction by L-isoproterenol and only partially inhibited induction of pro-CR$^+$ cells by L-isoproterenol. This suggests that under the conditions of our assays the observed effect of propranolol is predominantly receptor-specific and is not due to non-specific membrane effects or to PDE activation (18).

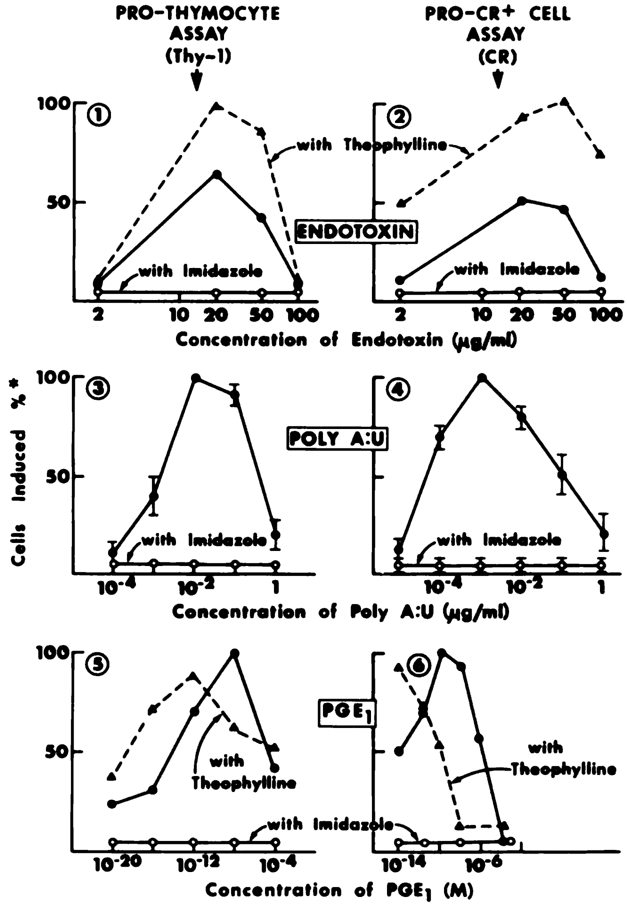

FIG. 5. Induction by endotoxin, poly A:U and PGE$_1$. The pharmacological data shown conform to the interpretation that induction by these three agents also involves a cAMP-dependent mechanism. (Concentrations of imidazole and theophylline as in Fig. 4.).* Data standardized as percent maximal induction (see footnote to Fig. 2). (Mean values for four to five tests.)

(d) TESTS WITH OTHER INDUCING AGENTS (Fig. 5). Endotoxin (panels 1 and 2) caused induction in both assays, maximal at 20 μg/ml and less at higher concentrations; (concentrations above 100 μg/ml were toxic). Induction was fully inhibited by imidazole (10^{-7} M), and was enhanced by theophylline (10^{-3} M). Lipid A gave similar results. Poly A:U (panels 3 and 4) gave optimal induction at a concentration of 10^{-2}–10^{-3} μg/ml, both inductions being fully inhibited by imidazole (10^{-7} M).

Prostaglandin PGE$_1$ (panels 5 and 6), which is linked to adenylate cyclase activation in various systems (18), including mouse lymphocytes (20), gave maximal induction of prothymocytes at a concentration of 10^{-8} M and of pro-CR$^+$ B cells at 10^{-10} M. Induction was inhibited by imidazole (10^{-7} M). Theophylline (10^{-3} M) enhanced induction by suboptimal concentrations of PGE$_1$, and inhibited induction by optimal or higher concentrations.

VII. *Features of Late B-Cell Differentiation: The PC$^+$ Phenotype*. PC is the

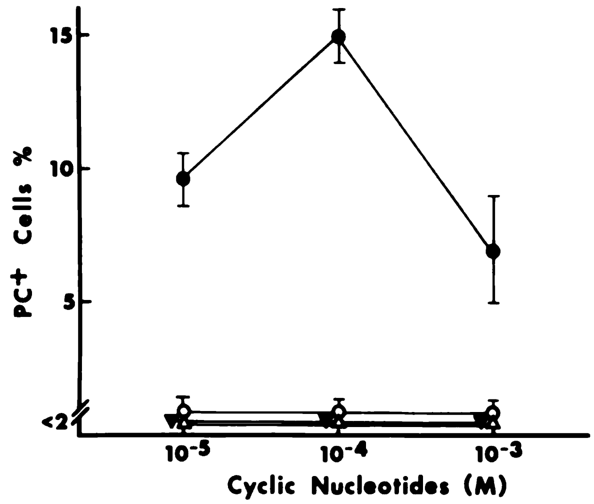

FIG. 6. Cyclic nucleotides: dose-response curves for induction of PC expression by cAMP (DBcAMP) and cGMP (8-bromo-cGMP) on Peyer's patch cells. Note: (*a*) induction by cGMP at lower concentrations is inhibited at higher concentrations, and (*b*) there is no induction by cAMP throughout the range of concentrations tested. (Mean values of four tests.) For B6-PC$^+$: cGMP ●, cAMP ○; for B6 cells (PC$^-$ congenic control): cGMP ▲, cAMP △.

last of the known surface markers expressed during sequential B-cell differentiation (6). The penultimate B-cell population, required for assaying PC induction, is Ig$^+$:CR$^+$:PC$^-$. (We find that this population contains cells that are not immediately PC-inducible, presumably either because they have not completed an intermediate differentiative step or because they belong to a subset committed to a different terminal program.) The inducible population for the PC system was Peyer's patch cells prepared from mice treated with *C. parvum*.

(A) CYCLIC NUCLEOTIDES. In contrast to early T- and B-cell induction (Section VI, above), the terminal phenotype PC was induced by cGMP, not cAMP. The dose-response relations (Fig. 6) show inhibition of induction with high concentrations of cGMP, as was the case with cAMP in early T- and B-cell inductions (Fig. 3). Similarly cAMP inhibited induction of PC by cGMP, as cGMP inhibited cAMP-mediated early T- and B-cell induction (Fig. 3).

(B) PHARMACOLOGY. If PC-induction is mediated by cGMP, the acetylcholine analog carbachol should act in this system as an inducer rather than an inhibitor (which it is in early T- and B-cell induction assays [(Fig. 3, panels 3 and 4]). Fig. 7 indicates that this is so.

(C) THYMOPOIETIN. The particular importance of thymopoietin is its claim to be a selective inducer of T-cell differentiation in the dual assay (see above, and reference 8). We therefore paid much attention to its action in the PC induction assay, especially because we had seen that thymopoietin partially inhibits CR$^+$ B-cell induction by cAMP or ubiquitin (data not shown), and totally inhibits induction of Ia (21). As Fig. 7 shows, a concentration of thymopoietin that is optimal for T-cell induction (Fig. 2) completely inhibited induction of PC by carbachol. A possible explanation is that thymopoietin is an inducer of PC, and that its ostensible suppression of induction by carbachol might be attributed to high-dose inhibition (thymopoietin plus carbachol) which

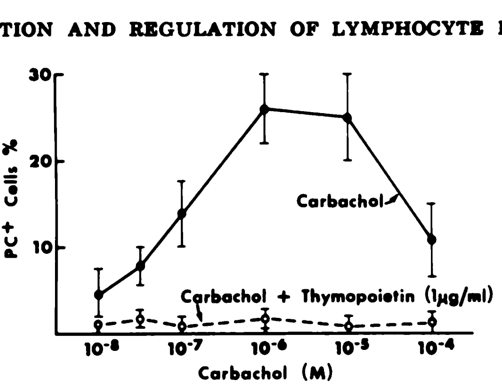

FIG. 7. Effect of thymopoietin (1 μg/ml) on induction of PC expression by carbachol. Note: thymopoietin inhibits induction by carbachol over the entire dose-response range. (Mean values of five tests.)

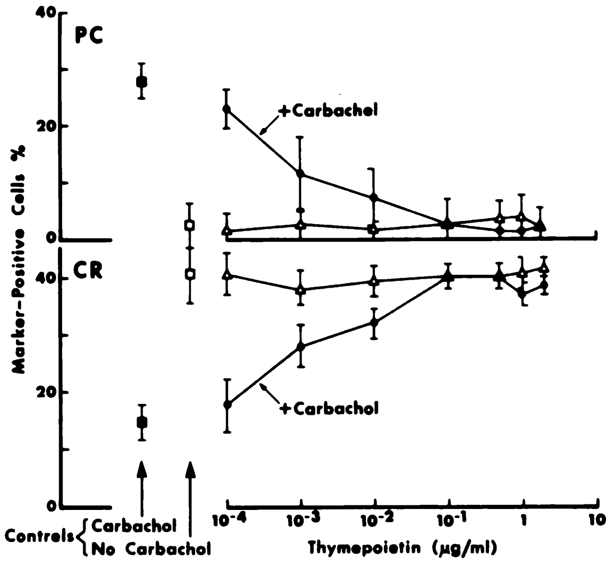

FIG. 8. Dose-response data for the effects of thymopoietin on carbachol-induced PC expression (upper panel), and on carbachol-induced reduction of CR expression (lower panel). These data illustrate that thymopoietin neither induced the expression of PC nor diminished the expression of CR, but suppressed both these effects of carbachol. (Mean values of three to eight tests.)

is documented for other inducers (Figs. 4 and 5). But this is not so, because thymopoietin should then enhance PC induction by a suboptimal concentration of carbachol, which it does not (Fig. 7). We confirmed this by assaying thymopoietin, over a wide range of concentrations, both alone and in combination with an optimal concentration of carbachol (10^{-6} M), for its effects on induction of PC expression. The upper panel of Fig. 8 shows that thymopoietin did not induce PC expression at any concentration tested, and that at no point did thymopoietin have any effect other than inhibition of PC induction by carbachol. The lower panel of Fig. 8, which refers to concomitant assays for CR expression with the same Peyer's patch cell suspension, shows that thymopoietin alone had no effect on the expression of CR, but that it prevented the fall in proportion of CR^+ cells that is characteristic of carbachol. We think it likely that PC expression and CR reduction are inversely correlated phenotypic changes affecting one cell set as it differentiates (though this wants proof). But regardless of mechanism these results signify that receptors for thymopoietin are present on $Ig^+:CR^+:PC^-$ cells.

Discussion

We judged CR, scored in the presence of EDTA, to be the most unequivocal routine marker for early B cells, and PC the obvious choice for late B cells. For T cells, Thy-1 is the best routine early marker. There is no well-authenticated late immunogenetic marker, comparable to PC, for T cells. But there are useful data on regulation of T-cell functions, to represent late phases of T-cell differentiation (20, 22, 23; reviewed in 24, 25), analogous to data for B-cell functions (26).

The principle of the dual assay, in which the induction of two different cell populations is observed simultaneously, has proved invaluable. It has allowed an explanation of why an assortment of agents, many seeming physiologically incongruous and with varied pharmacological actions, can induce prothymocytes in vitro. It is now amply substantiated that prothymocytes and homologous cell sets are preprogrammed for particular phenotypes, and that only thymopoietin, among the variety of prothymocyte-inducing agents tested, acts selectively to induce this particular committed cell set.

The application of dual assays in other induction systems now reveals that thymopoietin probably has a more comprehensive role in lymphocyte regulation than was at first supposed. Thymopoietin's selective inductive action might have been explained by restriction of thymopoietin receptors to the prothymocyte. Evidently this is not so. The ability of thymopoietin to inhibit induction of PC (and Ia [21]) completely, and of CR partially (data not included), is no less striking than its selective induction of prothymocytes. Thus thymopoietin receptors are evidently present on B cells also, though presumably geared in this case to a biochemical circuit that inhibits rather than initiates induction. Ubiquitin is a nonselective inducer which evidently engages a different receptor, doubtless with its own biochemical linkage, which accords with other experiments in which we have found that ubiquitin can override the inhibition of CR induction produced by thymopoietin.

Can all these facts relating to initiation and inhibition of induction be plausibly related to a general scheme of differential regulation by intracellular cyclic nucleotides?

First, induction of Thy-1 and of CR can be viewed as early steps in the differentiation of T and B cells, respectively, and both are evidently linked to cAMP. Secondly, induction of T-cell functions (24, 25), and induction of PC (and of B-cell functions; reviewed in 26), can be viewed as late steps in the differentiation of T and B cells, respectively, and these evidently are linked to cGMP. This conforms to a scheme in which the exogenous cyclic nucleotides (which are inducers in their own right), and similarly the non-specific agents which affect levels of intracellular cyclic nucleotides, have contrasting effects on the early and late differentiative steps referred to, and make no distinction between T cells and B cells. Thymopoietin on the other hand has opposite effects on T and

B-cell differentiative steps, both early and late.[2]

We do not infer that the nonselective induction observed in dual assays in vitro has no physiological counterpart in vivo. On the contrary, this may be a pointer to regulators which are necessary to govern lymphocyte populations as a whole in vivo. Possibly the serum factor facteur thymique serique (27, 28), which has properties similar to ubiquitin in vitro, including sensitivity to inhibition by adrenergic blocking agents (29), could qualify for such a role. Thymopoietin, in this frame of reference, plays the part of a differential regulator of lymphocyte sub-populations.

Differential regulation of lymphocyte sub-populations by thymopoietin can be considered in terms that are familiar in the context of hormone-receptor interactions (Sonenberg and Schneider; 30) and which include – (*a*) degradation of the hormone into inductive and other fragments, at the plasma membrane or in the cytoplasm, (*b*) alternative modes of coupling of the receptor to one or another effector mechanism in the plasma membrane, and (*c*) ensuing activation of particular effector mechanisms and intervention of second messengers.

There are good precedents, especially in neurotransmission, for the production of contrasting effects on cellular cAMP and cGMP by the same agent acting on cells of different sets (31, 32). Ontogeny in the lymphoid system (and doubtless generally) requires the balanced output and inter-regulation of multiple cell sets that emanate from a common stem set and that become separately but coordinately programmed for cooperative functions. Thymopoietin, with its reciprocal effects on facets of T and B lymphocyte differentiation that depend on cyclic nucleotides, appears well fitted for the role of differential regulator, and this affords the most plausible explanation for the inclusion of thymopoietin receptors in the phenotypes of different classes of lymphocyte.

Summary

Results with a dual assay, for the induction of Thy-1$^+$ T cells and of CR$^+$ B cells from marker-negative precursors, confirm that thymopoietin is at present the only known selective inducer of prothymocytes. In contrast, various inducers, including ubiquitin, are active in both assays. Pharmacological evidence indicates that there are different cellular receptors for ubiquitin and thymopoietin.

Prothymocytes and pro-CR$^+$ B cells compose two distinct populations in bone marrow and spleen; their distribution in density gradients is different, and elimination of either population enriches the other proportionately.

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[2] Measurements of cellular cAMP in the standard cell population used for induction have shown significant elevation with nonselective inducers (poly A:U, endotoxin, ubiquitin) but not so far with thymopoietin (R. G. Coffey, M. P. Scheid, and J. W. Hadden, unpublished observations). The reason for this may be purely technical, reflecting the smaller number of cells induced by thymopoietin. Because only limited enrichment of the prothymocyte population is as yet feasible, elevation of cAMP induced selectively in prothymocytes by thymopoietin may be too small to measure under present conditions of testing. We are investigating the use of α-Lyb-2 sera to eliminate B cells (in which we assume cAMP to be elevated by the nonselective inducers) and thus achieve adequate enrichment for prothymocytes. Indications that thymopoietin elevates cGMP in cell populations rich in mature peripheral T cells (23) conform to the argument developed further in this discussion.

There are no noteworthy differences between induction of these two populations in regard to (*a*) kinetics, (*b*) dependence on temperature and protein synthesis, (*c*) activation by cAMP, and (*d*) inhibition by cGMP. The opposite inductive effects of cAMP and cGMP were corroborated by the use of pharmacological agents that raise or lower the levels of intracellular cyclic nucleotides.

In contrast, a third induction assay, which monitors acquisition of the PC^+ surface phenotype, indicates that this differentiative step, the last known for B cells, is initiated by cGMP and inhibited by cAMP.

Induction of PC is also inhibited by thymopoietin, signifying that the inductive selectivity of thymopoietin is not due to restriction of its receptors to the T lineage cells. Rather it seems that receptors for thymopoietin occur also on PC-inducible and other B cells, although in this case geared biochemically to inhibition rather than expression of the succeeding gene program. This suggests a role for thymopoietin in the coordinated interregulation of lymphocyte classes, in addition to its better-known function as the thymic inducer of prothymocytes.

Present data conform to a general scheme in which the cyclic nucleotides cAMP and cGMP, and agents that affect intracellular levels of these mediators, influence reciprocally the early and late (functional) phases of lymphocyte differentiation as a whole, while thymopoietin influences reciprocally the differentiation of the B and T classes of lymphocyte.

We are indebted to Dr. Martin Sonenberg and Dr. John Hadden for their advice. The expert technical assistance of Jeannette Dilley, Regan Ihde, and Dennis Triglia is gratefully appreciated.

Received for publication 25 January 1978.

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THE SWITCH FROM IgM TO IgG SECRETION IN SINGLE MITOGEN-STIMULATED B-CELL CLONES

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B-lymphocyte mitogens, such as lipopolysaccharide (LPS) (1) or lipoprotein (2) stimulate murine B cells to growth and maturation to Ig secretion (3). Every third B cell of spleen is stimulated by LPS or by lipoprotein to grow to a clone of IgM-secreting, plaque-forming cells (PFC)[1] (4, 5). Mitogenic stimulation also results in the appearance of cells secreting IgG (6, 7). IgM- and IgG-secreting cells are detected by the protein A sheep erythrocyte-plaque assay (8). We determine in this paper the frequency of mitogen-stimulated B-cell clones which develop IgG-secreting, PFC under culture conditions which will let every growth-inducible B cell grow into a clone of cells (4, 9). Since we can limit the number of mitogen-reactive growth-inducible B cells to one per culture, we can investigate how many of the clones containing IgM-secreting PFC will develop IgG-secreting PFC, i.e. switch the class of Ig they produce, and how many clones develop IgG-secreting PFC only.

Materials and Methods

Animals. C3H/Tif/BOM 6–8 wk of age, were obtained from Dr. Bomholtgaard, Ry, Denmark. C57BL/6, DBA/2, and BALB/c mice, 6–8 wk of age, and Lewis strain rats, 4 wk of age, were from the Institut für Biologisch-medizinische Forschung AG., Füllinsdorf, Switzerland.

Cells, Mitogens, and Culture Conditions. Spleen cells were grown at concentrations indicated in the Results section in RPMI-1640 medium containing 2-mercaptoethanol (2-ME, 5×10^{-5}M), fetal calf serum (concentrations and batches indicated in the Results), and 3×10^6/ml of rat thymus filler cells in plastic tissue culture dishes or tubes (4, 5, 9).

Small, resting lymphocytes were purified from larger cells containing dividing and PFC (10) by 1 *g* velocity sedimentation (11).

LPS-S (EDTEN 18735 and S435/188049) was kindly prepared for us by Doctors C. Galanos and O. Lüderitz, Max-Planck-Institut für Immunobiologie, Freiburg i. Breisgau, West Germany and used at 50 μg/ml in culture. *Escherichia coli* lipoprotein, a gift from Dr. V. Braun, Mikrobiologie II, Universität Tübingen, Tübingen, West Germany, was used at 2 μg/ml (2).

Plaque Assay for Ig-Secreting Cells. For the detection of all cells secreting IgM or IgG a modified hemolytic plaque assay was used (8). Protein A-coupled SRC and rabbit anti-Ig (either anti-IgM or anti-IgG) antisera were used in the assay as developing antibodies together with properly diluted complement (Bio-Cult, Irvine, Scotland). Protein A used for the coating of SRC was obtained from Dr. H. Wigzell, Biomedicum, University of Uppsala, Uppsala, Sweden. Rabbit anti-mouse IgM antibodies were raised against purified myeloma MOPC 104E 19 S IgM (λ,μ).

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* Supported by the Swedish Cancer Society.

[1] *Abbreviations used in this paper:* B, bone marrow derived-, bursa equivalent; H-(μ,γ), heavy chain (μ,γ); Ig(M,G), immunoglobulin (M,G); LPS, bacterial lipopolysaccharides; 2-ME, β-mercaptoethanol; PFC, plaque-forming cells; SRC, sheep erythrocytes.

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Rabbit anti-mouse IgG antibodies were a mixture of antibodies raised against purified myeloma MOPC 21 IgG_1 (κ,γ) and against purified myeloma Adj-PC-5 IgG_{2a} (κ,γ_{2a}). Each antibody preparation was titrated for optimal developing capacity in the plaque assay (8), using myeloma cell lines producing the appropriate IgG subclasses. Since the two anti-IgG-antisera detected different populations of IgG-PFC (IgG_1 and IgG_{2a}), each of them was titrated separately and the mixture made up as a solution of both antisera at their independently optimal concentrations.

The subclass specificity of the two anti-IgG-antisera apparent in the protein A plaque assay was tested as follows: the anti-IgG_1-antiserum, at dilutions used for development of IgG_1-secreting PFC (tested with the X63AG8-myeloma cell line), did not develop plaques with 5563- and Adj-PC-5-myeloma cells (secreting IgG_2), with MOPC 315 cells (secreting IgA), with MOPC 104 E cells (secreting IgM), and with MOPC 41 and 46 cells (secreting K-L-chain). The anti-IgG_2 antiserum, at dilutions used for the development of IgG_2-secreting cells (tested with the 5563-myeloma cell line), did not develop plaques with the IgG_1-secreting X63-AG8-myeloma line nor with any of the other myeloma cells secreting IgA, IgM, or K-L-chains mentioned above.

Results

Mitogen-Stimulated Development of IgG-Secreting PFC in Cultures Containing Limiting Numbers of Reactive Precursors

FREQUENCY OF MITOGEN-REACTIVE B CELLS IN SPLEEN DEVELOPING TO CLONES OF IgG-SECRETING PFC. We have developed culture conditions with which each growth-inducible B cell will grow and maturate into a clone of Ig-secreting cells under the stimulatory influence of a mitogen (4, 5, 9). This was achieved by adding 3 × 10^6 thymus filler cells, either from syngeneic or allogeneic mice or from xenogeneic rats, into the culture medium. We can, therefore, dilute the number of reactive B cells until they become limiting in cultures. This allows us to determine frequencies of reactive B cells. According to Poisson's distribution, one reactive B cell is present in that number of cultured cells, which will let 37% of all cultures appear negative in a measured response. With such limiting dilution analyses we have previously determined that one LPS-reactive or one lipoprotein-reactive B cell yielding a clone of IgM-secreting PFC is present in three to four splenic B cells of 6–8 wk old C3H/Tif mice (5).

The results of limiting dilution analyses of splenic B cells of 6–8 wk old C3H/Tif and of C57BL/6J mice for the frequencies of precursor B cells yielding IgG-PFC is shown in Fig. 1. The linear digression of the curve, plotted as numbers of cultured cells against the logarithm of the fraction of nonresponding cultures, indicates that precursor B cells reactive to either LPS or to lipoprotein yielding IgG-PFC were limiting the cultures. Earlier results on the frequencies of reactive B cells yielding an IgM-PFC response (4, 5) could be confirmed (Table I). The frequencies of LPS or of lipoprotein reactive B cells in either C3H/Tif or in C57BL/6J-spleen cells yielding a clone of IgG-secreting PFC were one tenth of those yielding a clone of IgM-secreting PFC. In these in vitro cultures, therefore, and stimulated by the B-cell mitogens LPS or lipoprotein, not all mitogen-reactive B cells have the capacity to develop into IgG-secreting PFC. We determine below whether the IgG-secreting PFC clones originate from IgM-synthesizing and secreting clones or whether they never synthesize or secrete IgM during their clonal development after stimulation.

CLONAL DEVELOPMENT OF IgM-SECRETING AND IgG-SECRETING PFC WITH TIME OF STIMULATION. The development of single clones of reactive cells yielding

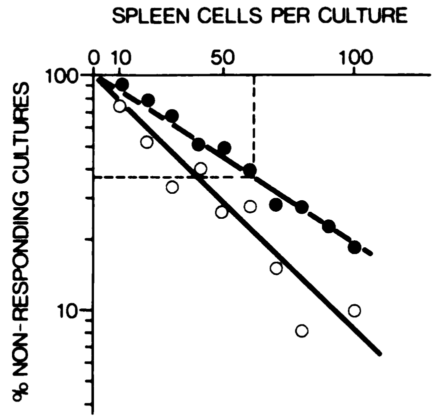

FIG. 1. Titration of LPS-reactive B cells yielding IgG-secreting clones. Abscissa indicate the number of input spleen cells per culture of C3H/Tif mice (●) or C57BL/6 mice (○). The cultures all contained a constant number of rat thymus cells (3 × 10^6 cells/ml) together with 50 μg/ml LPS. The assays for IgG-secreting PFC were done at day 7 of culture. The dotted line points to the number of C3H/Tif spleen cells per culture at which 37% of all cultures did not yield a response, i.e., which, according to Poisson's distribution, contained one mitogen-reactive B cell.

TABLE I

Frequencies of Mitogen-Reactive Splenic B Cells Yielding a Clone of IgM- or of IgG-PFC

Mouse strain	Mitogen	Absolute frequencies of mitogen-reactive B cells yielding clones of			
		IgM-Secreting PFC		IgG-Secreting PFC	
		LPS	Lipoprotein	LPS	Lipoprotein
C3H/Tif		1 in 3	1 in 4	1 in 30	1 in 40
C57BL/6J		1 in 2	1 in 3	1 in 25	–

IgM-secreting PFC or yielding IgG-secreting PFC could be followed by diluting the reactive B cells to near one per culture, i.e., to 6 C3H/Tif-spleen cells per culture for IgM-secreting PFC clones, and to 60 spleen cells per culture for IgG-secreting PFC clones. IgM-secreting PFC clones were assayed for 3 days between days 4 and 6 of culture, IgG-secreting PFC clones for 5 days between days 4 and 8 of culture. Table II summarizes the experimental data.

In agreement with our studies on the development of IgM-secreting PFC clones (4) the fraction of nonresponding B-cell cultures with time of stimulation remained constant, while the average number of IgM-PFC per culture increased steadily, doubling approximately every day. IgG-secreting PFC clones were practically not detectable at day 4 of growth, where IgM-secreting PFC clones were well detectable. IgG-secreting PFC clones began to appear from day 5 on, reaching a constant value for the fraction of nonresponding cultures at day 6. The average number of IgG-secreting PFC, however, continued to increase until day 7. This indicated that between days 6 and 7 of culture, IgG-secreting PFC

TABLE II

Development of IgM-Secreting PFC-Clones and of IgG-Secreting PFC Clones with Time of LPS-Stimulation under Culture Conditions Limiting the Number of Reactive B-Precursors to Near One

Assay (time of culture)	Number of positive cultures per total[*]	Fraction of negative negative (nonresponding) cultures	Average number of PFC per culture
IgM-secreting PFC clones: 40 cultures each at 6 C3H/Tif-spleen cells[‡] per culture			
Day 4	18/40	0.55	8.6
Day 5	18/40	0.55	15.0
Day 6	19/40	0.525	28.0
IgG[§]-secreting PFC clones: 40 cultures each at 60 C2H/Tif spleen cells[‡] per culture			
Day 4	2/40	0.95	0.9
Day 5	11/40	0.725	18.4
Day 6	18/40	0.55	29.5
Day 7	19/40	0.525	75.0
Day 8	18/40	0.55	75.0

[*] In medium containing 25% fetal calf serum.

[‡] Small cells purified from background PFC by 1 *g* velocity sedimentation.

[§] IgG$_1$ + IgG$_{2a}$.

TABLE III

Frequencies of Background PFC Secreting IgM or IgG$_{1+2}$ in Spleen of Different Inbred Strains of Mice

Mouse strain	Frequency[*] (per number of spleen cells)	
	IgM-Secreting	IgG$_{1+2}$ Secreting
C57BL/6J	1 in 800	1 in 7,000
C3H/Tif	1 in 150	1 in 1,800
BALB/c	1 in 180	1 in 1,200
DBA/2	1 in 400	1 in 4,500

[*] Average of five mice.

clones were growing in a constant fraction of all cultures.

This is only so when purified small, resting spleen cells are used for mitogenic stimulation. With unfractionated cells, also containing large, background PFC, a fraction of which secrete IgG, measurable IgG-PFC responses are detected early in culture as well. In fact, IgG-secreting, background PFC were found to amount to approximately 10% of the number of IgM-secreting, background PFC in 6–8 wk old mice of several inbred strains (Table III).

DEPENDENCE ON THE CONCENTRATION OF FETAL CALF SERUM IN CULTURE. We have previously found in experiments with cultures containing excess reactive B cells (9) that the length in time of development and the magnitude of the mitogen-induced IgG-secreting PFC response was dependent on the concentration of fetal calf serum in culture. We can now reinvestigate whether higher

TABLE IV

Dependence on the Concentration of Fetal Calf Serum of the Frequencies of LPS-Reactive B-Cells Developing to Clones of IgM-Secreting and to IgG-Secreting PFC Clones, and of the Size of these Clones, in Cultures Limiting the Number of Precursor B-Cells to Near One

Fetal calf serum concentration	Number of positive cultures per total	Fraction of negative (nonresponding) cultures	Average number of PFC per culture
%	IgM-secreting PFC clones: 24 cultures each, with 6 C3H/Tif spleen cells per culture		
2	2/24	0.91	1.5
5	14/24	0.41	26.7
10	14/24	0.41	59.0
20	15/24	0.38	59.5
30	15/24	0.41	56.0
	IgG-secreting PFC clones: 40 cultures each, with 60 C3H/Tif spleen cells per culture		
2	0/40	1.0	0
5	5/40	0.875	3.2
10	22/40	0.45	30.0
20	28/40	0.350	103.0
30	29/40	0.325	100.5

concentrations of fetal calf serum increase the frequencies of reactive B cells yielding an IgG-PFC clone and/or the size of the IgG-secreting PFC clones, and, thereby, the length of growth of these clones. Spleen cells from 6 to 8 wk old C3H/Tif mice were grown at 60 cells per culture in the presence of LPS and of different concentrations of fetal calf serum. Assays of all cultures were done at day 7 of culture. The responses of these spleen cells were controlled by measuring the IgM-secreting PFC response at six spleen cells per culture at the different fetal calf serum concentrations at day 5 of culture. Table IV summarizes the experimental results.

A constant fraction of all splenic B cells (six cells per culture) responded between 5 and 30% fetal calf serum with an IgM-secreting PFC response. The clone size indicated by the average number of IgM-secreting PFC per culture reached a constant number at 10%. Only 2% fetal calf serum was clearly not sufficient to let a constant fraction of all reactive B cells respond with measurable number of IgM-secreting PFC. Higher concentrations of fetal calf serum, between 20 and 30%, were needed in culture before a constant fraction of all spleen cells (60 cells/culture) responded with the development of IgG-secreting PFC. At those concentrations the size of the IgG-secreting PFC clones also reached a constant number of IgG-PFC per culture. Slightly lower frequencies of reactive B cells were measured with lipoprotein as stimulating B-cell mitogen (data not shown). These results indicate that there is no considerable recruitment of IgG-secreting cell clones from the resting pool late after stimulation, and therefore suggests that all the IgG-secreting clones which we detect at day 6 or 7 start to grow at time 0 of stimulation.

DISTRIBUTION OF THE NUMBER OF IgM-SECRETING PFC AND OF IgG-SECRETING PFC IN INDIVIDUAL CULTURES OF LPS-STIMULATED SPLENIC B CELLS. The absolute number of IgM- and IgG-secreting PFC was determined at a dilution of

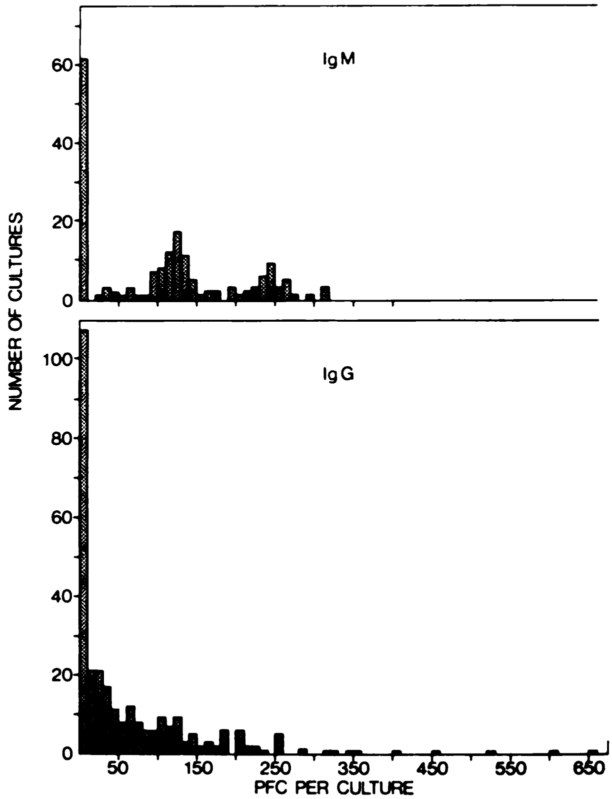

FIG. 2. Distribution of IgM- and IgG-secreting PFC in individual cultures of splenic B cells. Small spleen cells of C3H/Tif mice were distributed in individual cultures at six spleen cells per culture (for IgM responses) or at 60 spleen cells per culture (for IgG responses—in both assays yielding approximately 37% nonresponding cultures) with 50 μg/ml LPS and 3 × 10^6 thymus cells per ml. The total number of IgM-secreting PFC was determined at day 5 while the number of IgG-secreting PFC was determined at day 7 of culture.

LPS-reactive splenic B cells where approximately 37% of all cultures did not yield a PFC response. Small, resting splenic lymphocytes purified by velocity sedimentation and stimulated by mitogen show a constant frequency of responding cells when assayed for IgM- (4) and IgG- (Table II) PFC clones at different times of growth. During these times of clonal growth the absolute number of IgM-secreting PFC found in single cultures shows a discontinuous distribution (Fig. 2, top) which, according to Poisson's distribution, indicates that individual cultures contain either none, one, or two LPS-reactive B cells capable of developing into a clone of IgM-secreting PFC (4). These results suggest that all reactive B cells start to grow at time 0 of mitogenic stimulation, that all cells in a growing clone divide and that all dividing cells in that clone secrete IgM.

A similar analysis of the absolute number of IgG-secreting PFC developing in single cultures after LPS stimulation does not show a discontinuous distribution (Fig. 2, bottom) under conditions where the frequency of responding IgG PFC producing clones is constant. Taken together with our previous findings (4) that during the first 5 days of culture every cell in a mitogen-stimulated B-cell clone

TABLE V

Switch of IgM-Secreting B-Cell Clones to IgG Secretion

Number of cultures			Fraction of		
Tested in total*	Positive for IgM-‡ secreting PFC	Positive for IgG-§ secreting PFC	IgM-PFC-Positive cultures in total cultures	IgG-PFC-Positive cultures in total cultures	IgG-PFC-Positive cultures in all IgM-PFC-positive cultures
Exp. 1					
480	86	9	0.18	0.02	0.1
Exp. 2					
720	98	10	0.14	0.014	0.1

	Number of IgG-PFC-positive cultures	Fraction of IgG-PFC-positive cultures, also positive for IgM-PFC among all IgG-PFC-positive cultures
Exp. 1	9	1 (9 out of 9)
Exp. 2	10	1 (10 out of 10)

* Containing an average of one spleen cell per culture C3H/Tif/BOM, i.e. an average of 0.16 LPS-reactive B cells (4).

‡ Assay day 5, positive with more than 10 PFC per culture.

§ Assay day 7, positive with more than 6 PFC per culture.

divides and every dividing cell secretes sufficient IgM to produce a plaque, these results indicate that growing, IgM-secreting B cells within one clone can switch early or late, i.e., after different numbers of cell cycles, to IgG secretion.

CLONAL ORIGIN OF IGG-SECRETING PFC. The inhibition of mitogenic stimulation to IgG-secreting PFC development by anti-μ antibodies (13) had previously suggested that IgG-secreting PFC may originate from mitogen-sensitive precursor B cells, which have IgM on their surface. Stimulation of such surface IgM-positive precursor B cells by a B-cell mitogen might lead first to a clone of cells secreting IgM, which later, during its growth and maturation, switches to IgG secretion. These surface IgM-positive B precursors may, on the other hand, never secrete IgM, but develop directly to IgG-secreting PFC. This was tested in cultures limiting the number of precursor B cells yielding an IgM-PFC clone to one, assaying for IgM-PFC at day 5, and, in the same cultures, for IgG-PFC at day 7 of stimulation. According to Poisson's distribution 82–90% of all cultures will be nonresponding, 16.4–9% will contain one clone from one precursor, 1.6–0.45% two clones from two precursors, and 0.1–0.2% three clones from three precursors, if an average of 0.1–0.2 B-cell precursors is plated per culutre. According to our previous results (4), an average of 0.1–0.2 B-cell precursors yielding an IgM response is expected in an average of 0.6–1.2 spleen cells from C3H/Tif mice. Since only one tenth of all LPS-reactive B-cells developing into a clone of IgM-secreting PFC will yield an IgG-secreting PFC clone we expected every 50th and 100th culture to contain such an IgG-PFC clone and every 5th–10th culture to contain an IgM-PFC clone. Table V summarizes the results of two such experiments. Among the 1,200 cultures tested, 19 yielded an IgG PFC response. All these 19 cultures had previously contained IgM-secreting PFC.

The probability that the IgM and IgG-secreting PFC clones would have arisen from independent precursors in these experiments can be calculated by using Fisher's exact test of independence. These probabilities were found to be 3.4×10^{-7} and 4.0×10^{-9}, respectively. This shows that the IgG-secreting cells, which develop during the first 7 days of mitogenic stimulation in culture are all members of clones which secrete IgM at an earlier time of clonal growth.

Discussion

The present results show that the frequency of mitogen reactive B cells which develop into a clone of IgG-secreting cells is only about 1.5% of the total number of spleen cells under conditions where the frequency of precursors of IgM clones is 15%. This is a minimum estimate under our best possible conditions for clonal growth and maturation to Ig secretion of mitogen-stimulated B cells (4, 5). The data show that this frequency of B precursor cells yielding IgG-secreting clones (i.e., approximately 1 in 60 spleen cells) is constant between days 6 and 7 of culture. Therefore, little or no recruitment of IgG-secreting PFC clones occur at this time of culture. However, improved culture conditions allowing growth beyond day 7 of culture will show more conclusively whether or not development of new IgG-secreting PFC clones will occur more frequently in more cultures at later times of growth.

Switching of mitogen-activated B cells to IgG secretion can also be observed with lymph node and thoracic duct lymphocytes at the same relative frequencies compared to IgM (unpublished observations, reference 9). Since the majority of lymphocytes from these organs are thought to belong to the mature, committed antigen-sensitive population of cells we think that the clones which we observed switching from IgM to IgG secretion derive from mature, committed, resting antigen- and mitogen-sensitive small B lymphocytes.

Strikingly similar relative numbers of IgM- and IgG-producing cells are obtained when background PFC in spleen are measured (see Table III). Again, the number of IgG-secreting background PFC is approximately one tenth of the number of IgM-secreting cells. It points to the possibility—previously discussed by us (14)—that background PFC are representative samples of the repertoire of mitogen-reactive precursors of a given lymphocyte population.

The results presented herein also demonstrate that IgG-secreting PFC clones, although only constituting 10% of the IgM-PFC clones, all arise in those cultures where previously an IgM secreting clone had developed. This finding can only be explained by an intraclonal "switch" in the class of Ig secreted by some of the cells in the clone. The alternative interpretation, namely that IgM- and IgG-secreting clones arise from distinct precursors but activation of IgG precursors necessarily requires the presence of an IgM-secreting clone does not fit our observations. Thus, if this were the case, the frequency of detectable IgG-secreting clones should be higher when every culture contains at least one IgM-secreting clone (as in the experiment shown in Fig. 1) than when over 80% of the cultures contain no such IgM-secreting clone (as in the experiments shown in Table V). This, however, is not the case since in both conditions around 1/60 cells gave rise to an IgG-secreting clone. Such a switch in the class of Ig secreted cells has already been suggested by others using different experimental systems (15–18).

The absolute numbers of PFC secreting either IgM or IgG in single cultures containing on the average one mitogen reactive B-cell precursor for the development of IgM- or IgG-PFC clones show different distributions. Numbers of IgM PFC among individual clones show a discontinuous distribution compatible with a Poissonian assortment of mitogen-reactive precursors and doubling times of 18 h from time 0 of stimulation, thus indicating that all cells in a growing clone divide and that all dividing cells secrete IgM for the first 5–6 days of culture (4).

The absolute numbers of IgG-PFC in individual clones, on the other hand, show a continuous distribution. Since all IgG-secreting PFC arise from members of IgM-secreting clones, the detected IgG-PFCs are descendants of cells which all start to grow at time 0 of stimulation. Furthermore, the frequency of IgG-secreting PFC clones remain constant under our conditions which indicates that no recruitment of IgG-secreting clones occur after day 5 or 6 of stimulation. Under such conditions a continuous distribution of the absolute numbers of IgG PFC is best compatible with a switch occurring at different times after stimulation by mitogen, but before day 6 of culture, in different individual clones of growing cells. Our present data cannot give any information on whether switching from IgM to IgG secretion happens only once in one cell of a growing B-cell clone, or can repeatedly occur in different cells of one clone.

Our finding that only 10% of the mitogen-induced IgM-secreting PFC clones to IgG secretion is in agreement with previously published results obtained in vivo. By analyzing the clonal products of precursor cells responding to various antigens it was found that between 10 and 14% (15, 17) of the IgM-secreting clones also contained IgG-secreting cells by day 6 after stimulation. Furthermore, it was found that few or no IgG-secreting clones developed independently of IgM-secreting clone (17).

The low frequency of IgM clones which switch to IgG secretion in our experiments is compatible with the low or undetectable IgG production found in the immune response to thymus-independent antigens (19). The classical T-cell dependence of IgG responses may result from the postulated existence of a separate subset of B cells responding to T-cell derived factors (20–22). Such a subset could then partially be distinct from the subpopulation of B cells reactive to the mitogens LPS and lipoprotein in that these B-cell subpopulations show a much higher frequency of IgM secreting clones switching to IgG secretion.

The experiments by Nossal et al. (15) and Sterzl and Nordin (17), however, indicate that the same minor fraction, i.e. 10–15%, of all stimulated IgM-secreting B-cell clones will switch to IgG secretion. It is, therefore, conceivable that, for all known ways of B-cell stimulation – T-independent or T-dependent – only 10% of the reactive precursors are committed to switch to IgG-secretion upon stimulation. The final relative number of IgM- and IgG-secreting cells and, thus, the numbers of secreted molecules in serum, must then depend on regulatory events which occur after the induction of the originally responsive precursors and which alter the relative number of IgM to IgG-producing cells from 10:1 to some other ratio. T-dependent responses which apparently favor high IgG-responses, particularly upon repeated challenge, must, therefore, favor the propagation of those 10% reactive B-cell clones which can switch to IgG secretion.

Summary

The frequency of mitogen-reactive B cells yielding an IgG plaque-forming cell (PFC) response has been determined in vitro by limiting dilution analysis under culture conditions which allow every growth-induced B cell to grow and mature into a clone of Ig-secreting cells. The frequencies of lipopolysaccharide (LPS)- and lipoprotein-reactive precursors for IgG-secreting cells in the spleen of 6–8 wk old C3H/Tif and of C57BL/67 mice were found to be between 1 in 30 and 1 in 40 B cells and, therefore, only one tenth of the frequencies of mitogen-reactive precursors of clones secreting IgM.

All IgG-secreting cells developed by switching in clones which previously contained IgM-secreting cells. This was shown in two experiments where the total number of mitogen-reactive precursor yielding IgM-secreting cell clones was limited such that 82 or 90% of all responding cultures originated from one precursor. Thus, of 480 cultures in the first and 720 cultures in the second experiment, 86 and 98 cultures were found positive, yielding IgM-secreting cells at day 5. When the same cultures were assayed at day 7 for IgG-secreting cells 9 and 10 cultures were found positive. All 19 cultures with IgG-secreting cells previously had contained IgM-secreting cells.

The probability that IgG-secreting cells and IgM-secreting cells would have arisen from independent precursors can be calculated using Fisher's exact test of independence. For the two experiments those probabilities are 3.4×10^{-7} and 4.0×10^{-9}. Since we have previously shown that each cell in a mitogen-stimulated, growing B-cell clone divides, and that each dividing cell secretes Ig, we conclude from these experiments that the large majority – in our experiments all – of the IgG-secreting cells in mitogen-stimulated B-cell clones develop by switch from IgM-secreting cells. IgG-secreting cells develop either early or late during growth of a single IgM-secreting cell clone. The switch to IgG secretion, therefore, is not fixed in the time of clonal growth after mitogenic stimulation.

The able technical assistance of H. Campbell, J. Monckton, and M. Tuneskog is gratefully acknowledged. We thank Dr. C. Steinberg for help with statistical analysis of the data.

Received for publication 25 January 1978.

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CELLULAR AND MOLECULAR REQUIREMENTS FOR X-LINKED, HAPTEN-SPECIFIC B-CELL BLOCKADE IN CBA/N MICE

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CBA/N mice, a mutant CBA subline, are of particular interest for studies of the requirements for B-cell activation since they harbor an X-linked B-cell defect (1). The defect is characterized by low levels of circulating IgM, reduced numbers of splenic B cells, and a total failure of existing B cells to mount immune responses to certain thymic-independent antigens, e.g., haptenated Ficoll derivatives (2–5). Those B cells present in CBA/N spleens are characterized by a generally increased density of cell surface IgM (6). In addition, CBA/N mice have an abnormally high ratio of mu chain to delta chain homolog on their B-cell surfaces (7). In this respect, the Ig receptor density of CBA/N B cells appears to resemble the Ig density on B cells of neonatal and very young mice (8). Since the usual progression from mu chain to delta chain homolog expression on these cell surfaces is greatly delayed in CBA/N mice and in the hybrid male progeny of CBA/N females, Scher et al. have proposed that the CBA/N defect might represent an arrest in B-cell maturational development (6, 7).

CBA/N mice and their affected hybrid male offspring are also profoundly sensitive to selective B-cell blockade (9). Several other animal models exist in which the injection of a haptenated thymic-independent antigen can block a subsequent response to a thymic-dependent antigen bearing the same hapten (10–14). The marked blockade susceptibility of CBA/N mice is transmitted via the X chromosome, since all the male hybrid offspring of CBA/N females are affected. The hybrid female offspring are not affected at dosage levels of haptenated polysaccharide up to three orders of magnitude above those which can yield virtually total hapten-specific blockade both in the CBA/N parents and in the hybrid males (9).

The present study was designed to explore the cellular and molecular conditions necessary for production of this selective blockade. Several haptenated polysaccharide agents were examined for their relative effectiveness in producing blockade, and a wide range of efficiencies was observed. In addition, the effect of prior immune status on blockade susceptibility was investigated. The generation of hapten-specific immune memory to a thymic-dependent antigen virtually abolishes the inherent susceptibility of CBA/N mice to selective hapten-polysaccharide-mediated blockade. Moreover, the imposition of blockade on secretory B cells in defective mice appears to have little if any effect on their subsequent development of B memory cell populations.

Materials and Methods

Animals. CBA/N, C3H/HeN, CBA/CaHN-T6, and F_1 hybrid mice derived from these inbred strains were obtained from the Small Animal Production Section of the National Institutes of Health, Bethesda, Md. The F_1 hybrids were prepared by breeding CBA/N female mice with C3H/HeN or with CBA/CaHN-T6 males. All mice used were between 6 and 15 wk of age. The CBA/N mice (formerly designated CBA/HN) are a mutant subline of CBA mice, and their origin has been previously described (1).

Immunogens. Keyhole limpet hemocyanin (KLH)[1] was purchased from Schwarz/Mann, Div. Becton, Dickinson & Co., Orangeburg, N. Y. Ficoll, a synthetic polymer of sucrose with an average mol wt of 400,000, was obtained from Pharmacia Fine Chemicals, Piscataway, N. J. Pneumococcal polysaccharide, type III (SIII), was a gift from Dr. Phillip J. Baker, National Institute of Allergy and Infectious Diseases. The enlarged hapten, *N*-(2,4-dinitrophenyl)-β-alanylglycylglycine, (DNP), was synthesized as its tert-butyloxycarbonyl hydrazide as previously described (15). This reagent was converted to its reactive acyl azide (16) and coupled to *N*-(2-aminoethyl)-carbamylmethylated Ficoll (AECM-Ficoll) as described by Inman (17). The pH of the reaction mixture was adjusted to ≅ 8.5 with 1 N KOH after addition of the hapten azide. The degree of conjugation was calculated from dry weight and optical absorbance measurements (17) to yield DNP_{59}-$AECM_{78}$-Ficoll, or simply, "DNP_{59}-Ficoll." The subscript refers to the average number of haptenic groups coupled to 400,000 daltons of the original Ficoll.

A portion of the naturally occurring carboxyl functions of pneumococcal polysaccharide, SIII, was converted to *N*-(2-aminoethyl) amide groups, and this modified polysaccharide was then conjugated with the reactive DNP azide as previously described (9). Spectrophotometric analysis (17) revealed 52 DNP groups bound to 400,000 daltons of polysaccharide. This product was designated DNP_{52}-SIII.

DNP_{22}-KLH was prepared as described by Inman, et al. (16). Here, subscripts refer to the number of haptenic groups per 100,000 daltons of original KLH. Ficoll, conjugated with *N*-ε-dinitrophenyl-L-lysine (with no tripeptide), was a gift from Dr. Philip R. B. McMaster, Bureau of Biologics. It was prepared by a cyanuric chloride coupling procedure which McMaster et al. have published (18). For convenience, this smaller-hapten derivative is designated as dnp_{25}-Ficoll. All antigens and blocking agents were injected intraperitoneally (i.p.).

Hapten-Conjugated Erythrocytes. Sheep erythrocytes (SRC) were obtained in sterile Alsever's solution from the National Institutes of Health Animal Production Unit. SRC, pooled from six to eight sheep, were conjugated with the tripeptide-enlarged DNP hapten under optimal conditions according to the method of Inman et al. (16).

Cell Suspensions. Spleens were obtained from mice at sacrifice, minced in cold Hanks' balanced salt solution, and cell aggregates were disrupted by aspiration through a Pasteur pipette. The cell suspensions were passed through a thin layer of glass wool to remove fibrous connective tissue, washed in Hanks' balanced salt solution, and counted on a model B Coulter counter (Coulter Electronics Inc., Hialeah, Fla.).

Plaque-Forming Cell Assays. Suspensions of spleen cells from individual mice were assayed for DNP-reactive antibody-secreting cells by a modification of the Jerne hemolytic plaque technique as previously reported (19). All recorded plaque-forming cell (PFC) values have been corrected for coexisting direct PFC background against SRC. Data have been expressed as the mean $\log_{10}$ PFC/spleen ± SEM. Student's *t* test was employed to evaluate differences between groups of mice. Differences were considered to be significant when *P* values of ≤ 0.05 were obtained.

Results

The Effect of Varying Hapten Derivatization Levels on the Efficacy of Specific Hapten-Polysaccharide-Mediated Blockade. $AECM_{78}$-Ficoll was conjugated

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[1] *Abbreviations used in this paper:* AECM, Aminoethylcarbamylmethyl; dnp, the 2,4-dinitrophenyl hapten; DNP, the tripeptide-enlarged hapten, *N*-(2,4-dinitrophenyl)-β-alanylglycylglycine; i.p., intraperitoneally; KLH, keyhole limpet hemocyanin; PFC, plaque-forming cells; SIII, pneumococcal polysaccharide, type III; SRC, sheep erythrocytes.

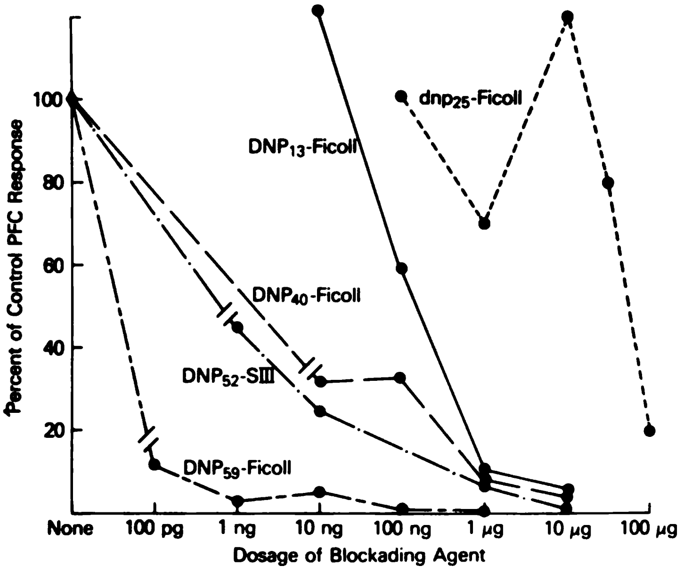

FIG. 1. Dose-response relationships in haptenated polysaccharide-mediated blockade. CBA/N mice were immunized with 50 μg of DNP_{22}-KLH i.p. 2 h after receiving varying i.p. dosages of haptenated polysaccharide blocking agents. Each dose-response curve represents the results for a single experiment; each point represents the geometric mean of the direct splenic DNP-reactive PFC response for 5 mice. The results have been normalized by assigning a value of 100% to the unsuppressed control group for each experiment to allow for comparisons between experiments.

with the tripeptide-enlarged DNP hapten at each of three derivatization levels, and these preparations were compared in dose-response experiments for their capacity to produce hapten-polysaccharide-mediated selective B-cell blockade. DNP_{52}-SIII and small hapten dnp_{25}-Ficoll were also employed in order to evaluate their blockade-invoking capacity. The data obtained are presented in Fig. 1. The normalized dose-response curves show the relative capacity of these haptenated polysaccharide agents to block the 4th-day direct PFC response to DNP_{22}-KLH in CBA/N mice.

DNP_{59}-Ficoll was the most effective blockading agent. It produced roughly 90% suppression of 4th-day direct PFC after injection of as little as 100 pg. Due to individual mouse variation, the first statistically significant blockade with DNP_{59}-Ficoll occurred at the 10-ng dosage level. This still amounted to a 5,000-fold net "signal advantage" per unit of weight over the 50 μg of DNP_{22}-KLH which was successfully blockaded. As previously observed in normal mouse strains by Desaymard and Waldmann (20), and Desaymard et al. (21), reducing the derivatization level of DNP-polymers profoundly reduced their efficiency in producing hapten-specific blockade. Also, dnp_{25}-Ficoll was particularly inefficient as a blockading agent. This implies that the tripeptide-enlarged form of the DNP hapten confers a more stable interaction with Ig recepters on target B cells, thus augmenting the blockade phenomenon. DNP_{52}-SIII, a linear saccharide polymer, was intermediate between DNP_{59}-Ficoll and DNP_{40}-Ficoll in its blockade-inducing effectiveness. This indicates that blockade is not produced exclusively by multi-branched polymers such as the haptenated Ficoll derivatives.

TABLE I

Failure of AECM-Ficoll to Produce Blockade of the 4th Day Direct PFC Response to DNP_{22}-KLH in CBA/N × C3H/HeN F_1 Mice

Group number* and sex	Blocking agent‡	Immunizing antigen	Mean log direct DNP-reactive PFC/spleen ± SE (geometric mean)	
			Experiment A	Experiment B
I. Male	$AECM_{120}$-Ficoll	–	ND§	2.39 ± 0.05 (244)
II. Male	–	50 μg DNP_{22}-KLH	3.61 ± 0.11 (4,107)	3.19 ± 0.19 (1,548)
III. Male	$AECM_{120}$-Ficoll	50 μg DNP_{22}-KLH	3.78 ± 0.25‖ (6,047)	3.02 ± 0.13‖ (1,046)
IV. Female	$AECM_{120}$-Ficoll	–	ND	3.54 ± 0.03 (3,496)
V. Female	–	50 μg DNP_{22}-KLH	4.47 ± 0.14 (29,279)	4.11 ± 0.14 (12,983)
VI. Female	$AECM_{120}$-Ficoll	50 μg DNP_{22}-KLH	4.33 ± 0.41¶ (21,222)	4.25 ± 0.07** (17,787)

* Four or five mice per group.

‡ 500 μg per mouse in Experiment A and 1 mg per mouse in Experiment B, always at −1 h.

§ ND, not determined.

‖ Does not differ significantly from group II of the corresponding experiment; in both instances $P \geq 0.20$.

¶ Does not differ significantly from group V; $P \geq 0.30$.

** Does not differ significantly from group V; $P \geq 0.10$.

Attempts to Blockade with Nonhaptenated Ficoll. In principle, blockade of B-cell activation by DNP-Ficoll might occur directly through its specific interaction with surface Ig receptors. Or, DNP-Ficoll might block by interaction of its polysaccharide moiety with some disparate surface component specialized to receive signals from polysaccharides or other polymeric entities. According to this latter alternative, Ig receptors would serve only to concentrate haptenated polysaccharide agents at the B-cell surface, with the carrier polysaccharide then delivering the critical signal to the defective cell. A preliminary test of this hypothesis was attempted by injecting 500 μg of $AECM_{120}$-Ficoll into male or female hybrid mice just 1 h before immunizing them with 50 μg of DNP_{22}-KLH. Our findings are presented in Table I. As often occurs, the 4th-day direct splenic PFC responses to DNP_{22}-KLH were substantially higher in the female than in the male hybrid mice in both experiments A and B. However, $AECM_{120}$-Ficoll did not appreciably reduce the response to DNP_{22}-KLH in male or female hybrid mice in either experiment. A further test was carried out in parental CBA/N mice using $AECM_{83}$-Ficoll. Here the preliminary injection of this polysaccharide carrier was given 4 h before primary immunization with DNP_{22}-KLH, and the $AECM_{83}$-Ficoll dosage was increased to 2 mg (Table II). Although this latter dose represents a 200,000-fold higher dosage of Ficoll than the 10 ng of DNP_{59}-Ficoll which provided significant blockade in Fig. 1, no indication of DNP-reactive PFC blockade could be detected.

Further experiments were performed to test whether blockade might be achieved by employing both the hapten and the Ficoll carrier signals separately. The results are provided in Table III. Free tripeptide-enlarged DNP hapten was

TABLE II

Failure of $AECM_{83}$-Ficoll to Blockade the 4th Day Primary Immune Response to DNP_{22}-KLH in CBA/N Mice

Group number*	Blocking agent given at −4 h	Immunizing agent given at time zero	Mean log direct DNP-reactive PFC/spleen ± SE (geometric mean)
I.	–	50 μg DNP_{22}-KLH	3.53 ± 0.11 (3,392)
II.	2 mg $AECM_{83}$-Ficoll	50 μg DNP_{22}-KLH	3.57 ± 0.12‡ (3,776)
III.	1 mg $AECM_{83}$-Ficoll	50 μg DNP_{22}-KLH	3.48 ± 0.15‡ (3,050)
IV.	500 μg $AECM_{83}$-Ficoll	50 μg DNP_{22}-KLH	3.45 ± 0.09‡ (2,826)
V.	250 μg $AECM_{83}$-Ficoll	50 μg DNP_{22}-KLH	3.71 ± 0.15‡ (5,214)

* Five mice per group.

‡ Does not differ significantly from group I; $P \geq 0.15$.

TABLE III

Chemical Attachment of Hapten and Ficoll Required for Effective Blockade of Direct PFC Response to DNP_{22}-KLH in CBA/N Mice

Group number*	Blocking agents‡		Mean log direct DNP-reactive PFC/spleen ± SE (geometric mean)
	First	Second	
I.	–	–	4.50 ± 0.05 (31,761)
II.	–	10 μg DNP_{59}-Ficoll	3.32 ± 0.07§ (2,089)
III.	–	0.2 ml 10^{-3}M DNP-hydrazide‖	4.46 ± 0.15¶ (28,969)
IV.	100 μg $AECM_{120}$-Ficoll	0.2 ml 10^{-4}M DNP-hydrazide	4.53 ± 0.03¶ (33,947)
V.	1 mg $AECM_{120}$-Ficoll	0.2 ml 10^{-3}M DNP-hydrazide	4.63 ± 0.13¶ (42,312)
VI.	500 μg $AECM_{120}$-Ficoll	10 μg DNP_{59}-Ficoll	3.00 ± 0.12§ (991)

* Five CBA/N female mice per group; all mice in all groups were immunized with 50 μg of DNP_{22}-KLH, i.p.

‡ First and second blocking agents given 10 min apart at ≅2 h before immunization with DNP_{22}-KLH.

§ Differs significantly from group I; $P \leq 0.005$.

‖ The tripeptide-enlarged free hapten, N-(2,4-dinitrophenyl)-β-alanylglycylglycine Boc-hydrazide.

¶ Does not differ significantly from group I; $P \geq 0.10$.

ineffective by itself in producing hapten-specific blockade. Free hapten and free Ficoll carrier administered concomitantly were also ineffective, even when each was employed at a 100-fold higher concentration (group V) than its effective concentration when covalently combined (group II). In addition, 500 μg of

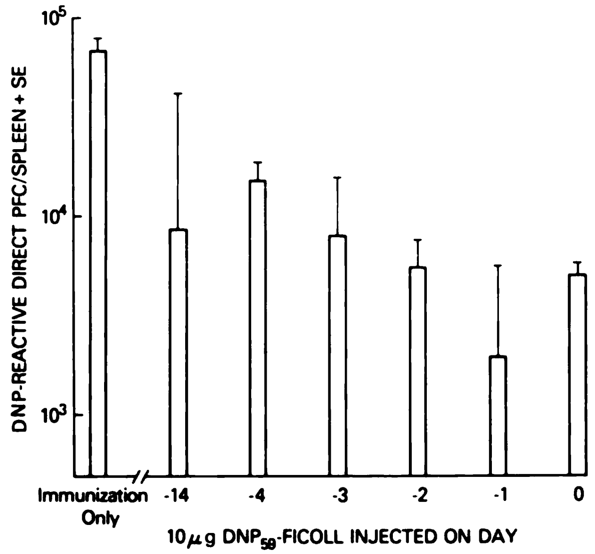

FIG. 2. Sustained blockade in CBA/N mice given DNP$_{59}$-Ficoll at various junctures before immunization with DNP$_{22}$-KLH. Groups of 5 mice each received 10 μg of DNP$_{59}$-Ficoll i.p. at intervals of up to 14 days before i.p. immunization with 50 μg of DNP$_{22}$-KLH. Splenic DNP-reactive direct PFC were determined 4 days after DNP$_{22}$-KLH immunization. Note that the degree of responsiveness is shown on a log scale.

AECM$_{120}$-Ficoll was ineffective in preventing the blockade imposed by only 10 μg of DNP$_{59}$-Ficoll. Accordingly, our findings indicate that the hapten and the carrier Ficoll must be chemically joined in order for hapten-specific blockade to occur.

Temporal Requirements for Production of Specific Hapten-Ficoll-Mediated Blockade. CBA/N mice were given 10 μg of DNP$_{59}$-Ficoll at various intervals before immunization with DNP$_{22}$-KLH and their direct splenic PFC responses were measured 4 days after immunization. The findings are depicted in Fig. 2 which discloses that the blockade phenomenon is obtained at least as efficiently with DNP$_{59}$-Ficoll delivered at −24 h as it is when the blockading agent is given at the time of immunization. DNP$_{59}$-Ficoll delivered at successively longer time intervals before immunization was progressively less effective in causing the blockade. When delivered 14 days before DNP$_{22}$-KLH immunization, 10 μg of DNP$_{59}$-Ficoll reduced the mean 4th-day PFC response to 18% of that in the control mice. Sufficient individual variation occurred in this group, however, so that the mean PFC reduction was not statistically significant. Thus, it appears that some mice have largely escaped from blockade after 14 days, but in others, the blockade remains relatively intact.

In another set of experiments, DNP$_{59}$-Ficoll was given at intervals ranging up to 48 h after immunization with 50 μg of DNP$_{22}$-KLH. The findings provided in Table IV, show that significant blockade was obtained in both CBA/N and F$_1$ male hybrid mice when DNP$_{59}$-Ficoll was given up to 4 h after DNP$_{22}$-KLH immunization. The findings also suggest that hybrid male mice may be susceptible to blockade for a longer time after immunization than their CBA/N parents.

TABLE IV

Blockade of the Splenic PFC Response to DNP_{22}-KLH by Post-Immunization Injection of DNP_{59}-Ficoll

Group number*	50 μg DNP_{22}-KLH given at time	10 μg DNP_{59}-Ficoll given at time	Mean log direct DNP-reactive PFC/spleen ± SE (Geometric Mean) [Percent of Control Response]	
			CBA/N Females	CBA/N × CBA/CaHN-T6 Males
		(h)		
I.	–	–	1.14 ± 0.46 (14)	1.06 ± 0.21 (12)
II.	0	–	3.36 ± 0.28 [100] (2323)	3.39 ± 0.17 [100] (2473)
III.	0	−2	2.53 ± 0.24 [14]‡ (341)	2.21 ± 0.42 [6]§ (164)
IV.	0	+4	2.41 ± 0.23 [11]‡ (260)	2.83 ± 0.14 [27]§ (691)
V.	0	+20	3.40 ± 0.13 [109]‖ (2549)	2.67 ± 0.49 [18]‖ (469)
VI.	0	+48	3.51 ± 0.12 [141] (3282)	3.25 ± 0.25 [73] (1818)

* Five mice per group; all injections were given i.p.

‡ Differs from group II; $P < 0.05$.

§ Differs from group II; $P < 0.025$.

‖ Does not differ significantly from group II of the corresponding experiment.

TABLE V

Failure of Prior Immunization with DNP_{22}-KLH to Prepare CBA/N × C3H/HeN F_1 Mice for Responsiveness to DNP_{59}-Ficoll

Group number* and sex	First immunization given on day 0	Second immunization given on day 15	Mean log DNP-reactive PFC/spleen ± SE‡ (geometric mean)	
			Direct PFC	Indirect PFC
I. Male	–	10 μg DNP_{59}-Ficoll	0.89 ± 0.36 (8)	1.13 ± 0.30 (14)
II. Male	50 μg DNP_{22}-KLH	–	2.56 ± 0.65 (367)	2.71 ± 0.68 (514)
III. Male	50 μg DNP_{22}-KLH	10 μg DNP_{59}-Ficoll	3.20 ± 0.13§ (1,607)	3.38 ± 0.09§ (2,448)
IV. Male	50 μg DNP_{22}-KLH	50 μg DNP_{22}-KLH	3.81 ± 0.34 (6,572)	4.05 ± 0.29 (11,431)
V. Female	–	10 μg DNP_{59}-Ficoll	4.86 ± 0.05 (73,958)	4.60 ± 0.05 (40,075)
VI. Female	50 μg DNP_{22}-KLH	–	3.47 ± 0.06 (2,994)	3.28 ± 0.03 (1,936)
VII. Female	50 μg DNP_{22}-KLH	10 μg DNP_{59}-Ficoll	4.37 ± 0.07‖ (23,815)	4.23 ± 0.07‖ (16,991)
VIII. Female	50 μg DNP_{22}-KLH	50 μg DNP_{22}-KLH	4.27 ± 0.28 (18,675)	4.46 ± 0.31 (29,087)

* Five mice per group.

‡ All spleens assayed on day 19.

§ Does not differ significantly from group II; $P > 0.10$.

‖ Differs from group V; $P < 0.0025$.

The Effects of Prior Immune Status on the Capacity to Respond to DNP_{59}-Ficoll. Although CBA/N mice and the hybrid male progeny of CBA/N females are unable to respond directly to DNP_{59}-Ficoll, the existence of a specific hapten-Ficoll-mediated blockade implies a significant interaction of their B cells with this polysaccharide agent. Conceivably, B cells from DNP_{22}-KLH-primed CBA/N mice might recognize the DNP_{59}-Ficoll signal as immunogenic. Thus, priming with DNP_{22}-KLH might induce in defective mice some DNP-reactive memory B cells which could then undergo activation on subsequent encounter with DNP_{59}-Ficoll.

Male and female hybrid mice were immunized with DNP_{22}-KLH and after 15 days, were challenged either with DNP_{22}-KLH or with DNP_{59}-Ficoll. The results of splenic PFC assays performed 4 days later are presented in Table V. Memory to DNP_{22}-KLH had been generated in both the male and female hybrid mice since their indirect PFC already exceeded their direct PFC on the 4th day post challenge (groups IV and VIII). In hybrid female mice, challenge with DNP_{59}-Ficoll caused both the direct and indirect PFC responses to be lower in DNP_{22}-KLH-primed than in unprimed animals (compare group VII with group V). It is important to note that for hybrid females, the immunogenicity of DNP_{59}-Ficoll is not neutralized by any circulating antibodies produced in response to prior immunization with DNP_{22}-KLH (compare groups VI and VII).

In male hybrid mice, the 4th-day direct and indirect PFC responses after challenge with DNP_{59}-Ficoll were both modestly elevated; but, the response levels did not differ significantly from the residual activity observed in control mice immunized 19 days earlier with DNP_{22}-KLH (compare group III with group II). It is conceivable that some defective F_1 male memory cells may be ultimately triggered by subsequent contact with DNP_{59}-Ficoll; however, the effect as currently observed falls far short of functional restoration.

Attempts to Impose Hapten-Specific Blockade on Memory Cell Populations in CBA/N Mice. Although generation of hapten-specific memory cell populations in defective mice conferred little if any capacity to respond directly to DNP_{59}-Ficoll, it was important to ascertain whether memory B cells, like precursor B cells, were susceptible to haptenated-Ficoll blockade. CBA/N mice were immunized with DNP_{22}-KLH, and 10 days later they were challenged with the same antigen either with or without coexistent DNP_{59}-Ficoll blockade. Our findings are recorded in Table VI. Again, indirect PFC outnumber direct PFC at 4 days after secondary challenge with DNP_{22}-KLH (compare group III with group II). DNP_{59}-Ficoll could not produce significant reductions in either the direct or indirect PFC responses of these memory cell-bearing defective CBA/N mice. Thus, the generation of memory cell populations allows escape from blockade in these defective mice even though it does not confer any capacity for direct responsiveness to DNP_{59}-Ficoll. Whether or not circulating antibody elicited by prior encounter with DNP_{22}-KLH could neutralize the blockading capacity of DNP_{59}-Ficoll is uncertain. Such antibody does not neutralize the immunogenicity of DNP_{59}-Ficoll in normal hybrid female mice (Table V).

The Effect of DNP_{59}-Ficoll-Mediated Blockade on the Development of B-Cell Memory to DNP_{22}-KLH. Although DNP_{59}-Ficoll virtually abrogates the 4th-day secretory B-cell response to DNP_{22}-KLH, it was not clear whether DNP_{59}-

TABLE VI

Failure to Impose Blockade on Memory Cell Populations in CBA/N Mice

Group number	Primary immunization	Blocking agent*	Secondary challenge‡	Mean log DNP-reactive PFC/spleen ± SE§ (geometric mean)	
				Direct	Indirect
I.	DNP_{22}-KLH‖	–	–	3.06 ± 0.11 (1157)	2.37 ± 0.08 (236)
II.	–	–	DNP_{22}-KLH	3.78 ± 0.12 (6020)	2.94 ± 0.11 (866)
III.	DNP_{22}-KLH	–	DNP_{22}-KLH	3.32 ± 0.14 (2082)	4.05 ± 0.03 (11,112)
IV.	DNP_{22}-KLH	DNP_{59}-Ficoll	DNP_{22}-KLH	3.21 ± 0.16 (1619)¶	3.82 ± 0.16 (6,545)¶

* 10 μg given 2 h before secondary challenge.

‡ 50 μg on day 10.

§ Assayed 4 days post secondary challenge. Five mice in each group.

‖ 50 μg given at time 0.

¶ Does not differ from group III; $P \geq 0.10$.

TABLE VII

*Failure of Blockade to Prevent Development of Memory Cell Populations in CBA/N Mice**

Group number	Primary blockade‡	Primary immunization	Secondary blockade‡	Secondary immunization	Mean log DNP-reactive PFC/spleen ± SE§ (geometric mean)	
					Direct	Indirect
I.	–	–	–	DNP_{22}-KLH	4.11 ± 0.13 (12,763)	2.86 ± 0.15 (732)
II.	DNP_{59}-Ficoll	DNP_{22}-KLH	–	–	3.18 ± 0.07 (1,507)	3.06 ± 0.08 (1,150)
III.	–	DNP_{22}-KLH	–	DNP_{22}-KLH	3.34 ± 0.06 (2,204)	4.43 ± 0.05 (27,180)
IV.	DNP_{59}-Ficoll	DNP_{22}-KLH	–	DNP_{22}-KLH	2.73 ± 0.13 (538)	4.30 ± 0.12‖ (19,830)
V.	DNP_{59}-Ficoll	DNP_{22}-KLH	DNP_{59}-Ficoll	DNP_{22}-KLH	3.33 ± 0.22 (2,157)	4.36 ± 0.06¶ (23,031)

* Tested by challenge at 30 days post primary blockade and immunization. Five mice per group.

‡ 10 μg DNP_{59}-Ficoll given 1 h before immunization with 50 μg DNP_{22}-KLH.

§ Assayed 4 days after challenge.

‖ Does not differ significantly from group III; $P < 0.20$.

¶ Does not differ significantly from group IV; $P < 0.40$.

Ficoll imposed an absolute blockade of all signals receivable by B cells from DNP_{22}-KLH. Accordingly, CBA/N mice were blockaded with DNP_{59}-Ficoll before an initial immunization with DNP_{22}-KLH, and 1 mo later they were challenged with DNP_{22}-KLH either with or without secondary blockade. Our findings are presented in Table VII. The defective CBA/N mice were blockaded on day 0 and challenged on day 30. They produced indirect PFC responses almost as high as those seen in control mice never subjected to blockade (compare groups III and IV). In addition, mice given a second blockade at the time of the 30 day challenge were now also completely refractory to blockade (groups III vs. V). Since we had previously shown that the effect of blockade is

TABLE VIII

*Failure of DNP_{59}-Ficoll-Mediated Primary Blockade to Affect the Secondary Immune Response to DNP_{22}-KLH in CBA/N Mice**

Group number	Primary blockade‡	Primary immunization§	Secondary blockade‖	Secondary immunization¶	Mean log DNP-reactive PFC/spleen ± SE** (geometric mean)	
					Direct PFC	Indirect PFC
I.	–	–	–	DNP_{22}-KLH	3.75 ± 0.27 (5659)	2.91 ± 0.11 (821)
II.	–	–	DNP_{59}-Ficoll	DNP_{22}-KLH	1.02 ± 0.44 (11)	0.47 ± 0.17 (3)
III.	–	DNP_{22}-KLH	–	DNP_{22}-KLH	3.20 ± 0.19 (1599)	3.78 ± 0.06 (6061)
IV.	DNP_{59}-Ficoll	DNP_{22}-KLH	–	DNP_{22}-KLH	3.14 ± 0.19 (1399)	3.82 ± 0.08‡‡ (6648)
V.	DNP_{59}-Ficoll	DNP_{22}-KLH	DNP_{59}-Ficoll	DNP_{22}-KLH	2.68 ± 0.11§§ (485)	3.50 ± 0.16‖‖ (3182)

* Tested by challenge at 8 days post primary blockade and immunization. Five mice per group.

‡ 10 μg DNP_{59}-Ficoll given on day 0.

§ 50 μg DNP_{22}-KLH on day 0 given 2 h after the DNP_{59}-Ficoll blockade.

‖ 10 μg DNP_{59}-Ficoll given on day 8.

¶ 50 μg DNP_{22}-KLH on day 8 given 2 h after the second DNP_{59}-Ficoll blockade.

** Assayed 4 days after challenge.

‡‡ Does not differ significantly from group III; $P < 0.40$.

§§ Differs from group IV; $P < 0.05$.

‖‖ Does not differ significantly from group IV; $P < 0.10$.

reduced with time after exposure to DNP_{59}-Ficoll (Fig. 2), it was important to recheck these findings at a time when memory cell populations might reasonably be expected to first appear. The same experiment was repeated, but the DNP_{22}-KLH challenge was given on day 8 rather than on day 30. Our findings are presented in Table VIII. Prior blockade with DNP_{59}-Ficoll did not prevent the full development of memory B-cell populations in response to the concomitant administration of DNP_{22}-KLH (groups III vs. IV). Efforts to produce secondary blockade at day 8 again failed to significantly reduce the indirect PFC response; the reduction of the direct PFC response, however, was marginally significant (groups IV vs. V). The overall effect stands in marked contrast to the effectiveness of DNP_{59}-Ficoll in blockading the primary 4th-day secretory PFC response to DNP_{22}-KLH. Apparently, blockade of 4th-day secretory B-cell responses is of little predictive value for blockade of memory B-cell responsiveness in CBA/N mice.

Discussion

The B Cell as the Probable Target of Hapten-Polysaccharide-Mediated Blockade. Our current findings focus attention on B cells as the probable direct targets of the X-linked hapten-specific blockade phenomenon. Polysaccharides carrying the tripeptide-enlarged DNP hapten were profoundly more efficient in producing blockade than was another polysaccharide bearing the traditional small form of the dnp hapten (Fig. 1). This implies a critical role for Ig receptors in the blockade phenomenon, as does the fact that increased haptenic density on the polysaccharide agents results in increased blocking efficiency. Multiple Ig receptors probably must be cross-linked by haptenated polymer for blockade to ensue, since unjoined free hapten and free Ficoll carrier (each provided at 100-fold their usual conjugated dosage) were unable to produce blockade. In addition, DNP$_{59}$-Ficoll given at the usual 10 μg dosage level could not be functionally displaced by prior injection of a 50-fold excess of nonhaptenated AECM$_{120}$-Ficoll (Table III).

It has been proposed (6) that the CBA/N B-cell defect in responsiveness to thymic-independent antigens may be expressed at any of three levels: (*a*) a failure to bind thymic-independent antigens on B-cell surfaces, (*b*) a failure to recognize thymic-independent antigens once they are bound on B-cell surfaces or (*c*) the absence of a unique B-cell subpopulation with the capacity to respond to thymic-independent antigens. Our present findings indicate that CBA/N mice do have B cells which can recognize polysaccharide antigens; the recognition event, however, leads uniquely to B-cell blockade rather than to B-cell activation. Thus, it is still possible that CBA/N mice may be intrinsically unable to recognize haptenated polysaccharides in any way that would lead to secretory activation of their defective B cells. In general, our present findings favor the view that in unprimed CBA/N mice, a unique B-cell subpopulation is functionally missing.

Possible Mechanisms of B-Cell Blockade. The presence of a B-cell defect in CBA/N mice is well established (3, 4). If indeed macrophages and T cells are not directly involved in the intrinsic defect, then hypotheses which emphasize B-cell characteristics may be constructed in an attempt to explain the functional defect. In principle, hapten-specific blockade of relevant B-cell clones could occur through critical interactions between Ig receptors on such B cells and (*a*) monovalent haptens, (*b*) the Ficoll carrier molecule[2] (here a disparate or unique secondary receptor specific for polysaccharide would be required to initiate the blocking signal[3]), (*c*) both of the above, i.e., separate recognition of both the hapten and the carrier moieties by the same cell, or (*d*) multivalent hapten. In this latter case, no receptor specialized for recognition of the polymeric carrier would be required, but a geometrically acceptable presentation of the haptenic epitopes to surface Ig receptors would suffice to effect blockade. In principle, this mechanism would allow blockade by haptenated polymers other than haptenated polysaccharides, as long as the critical requirements of epitope presentation geometry were met (22). Our current data appear to be supportive of either mechanism (*c*) or (*d*). If mechanism (*d*) is operative, then the requirements for critical epitope presentation can be about equally well met by either linear or multi-branched haptenated polysaccharide blocking agents (Fig. 1).

The joint signal provided by hapten-conjugated carrier could be either passive or active in nature. It is conceivable that the multiple site interaction which occurs between Ig receptors and multivalent haptenated Ficoll prevents dissociation of hapten from these Ig receptors, and thereby assures saturative preemption of most available receptors. Such a blockade mechanism would be passive in nature and would not necessarily require metabolic changes in the cell. At present, no adequate explanation can be offered for why cell capping and consequent shedding of antigen-antibody complexes would not provide for an early escape of B cells from such hapten-specific blockade.

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[2] Although current findings provide no support for these first two alternatives, final consideration of these possibilities is being temporarily held in abeyance since higher test concentrations of free hapten and/or free carrier can be attained in in vitro or cell transfer systems.

[3] Two sets of authors have recently reported the absence of unique differentiation antigens on spleen cells from CBA/N and/or hybrid male mice (25, 26). To the best of our knowledge, there are no reports claiming the presence of any additional unique antigens or receptors on lymphocytes from these B-cell defective mice.

The marked effectiveness of X-linked hapten-polysaccharide-mediated blockade, even at extremely low dosage levels (Fig. 1), seems consistent with an active role for haptenated polysaccharide blocking agents. In this instance, the joint signal provided by haptenated polysaccharide would produce distinct metabolic changes in the B cell. These changes would make the B cell refractory to subsequent thymic-dependent signals for secretory activation. Presumably refractoriness to secretory activation would not preclude memory cell activation.

A related concept has recently been proposed to account for B-cell activation in normal mice (22). According to this concept, an activation signal for normal B cells can be accomplished by the formation of "immunons" at B-cell surfaces. An "immunon" is formed when ≅12–16 Ig receptors are linked together by antigen in a spatially continuous cluster which can contribute to an activation signal. The number of geometrically stable "immunons" which must be formed on any given B-cell surface in order to cause its activation is unknown. Since many thymic-independent antigens are intrinsically nonmitogenic (5), and are thus presumably incapable of delivering an ordinary mitogenic second signal, the "immunon" concept is especially attractive.

The currently advanced alternative (which might apply only to defective CBA/N mice) requires only that a similar geometrically stable arrangement of cell surface Ig might be generated by haptenated polysaccharide agents. These blocking agents would then actively lead to a refractory B-cell state. For convenience, such a critical arrangement of cell surface Ig receptors might be termed a "toleron." Conceivably, the generally increased density of Ig surface receptors on the B cells of CBA/N mice (6) might predispose to "toleron" development.

Refractoriness of B Memory Cells to Hapten-Polysaccharide-Mediated Blockade. Although CBA/N mice appear to be delinquent in the development of mature IgM to IgD-type cell surface receptor relationships (7, 8, 23), prior immunization with a thymic-dependent antigen generates specific B memory cell populations. This maturational change is accompanied, no doubt, by marked changes in the representation and density of surface Ig receptors on the specifically affected B-cell clones (11, 12). Thus, some responding B cells may escape their usual prolonged infancy. These memory cells are apparently no longer susceptible to haptenated polysaccharide-induced blockade, but they have still not acquired any appreciable capacity for direct responsiveness to haptenated polysaccharide antigens (Table V). Why changes in Ig receptor density could lead to escape from susceptibility to blockade without recovery of some capacity for direct responses to polysaccharide antigens cannot be readily explained. One possibility is that circulating antibody generated at the time of memory cell induction with DNP_{22}-KLH could effectively intercept and neutralize the blockading capacity of DNP_{59}-Ficoll. In this case, the memory cells would not actually be subjected to blockade, and their failure to respond to challenge with DNP_{59}-Ficoll would simply represent maintenance of their innate unresponsiveness to that antigen.

The early observations of Baker et al. (24) extended by Schrader and Nossal (13) have demonstrated that even "end-stage" secretory B cells are susceptible to external stimuli imposed by surface-bound antigens. This phenomenon has been termed effector cell blockade, and Schrader has recently shown (14) that it is accomplished by multipoint interactions with Ig receptors. He has also shown that specific enzymatic interruption of such Ig linkages results in the reversibility of the delivered signal. The work of Klaus has emphasized that B precursor cells of normal mice are also susceptible (at quite high dosages) to specific hapten-polysaccharide-imposed blockade (10–12). Thus, it seems reasonable to assume that B precursors as well as B secretory cells might well be susceptible to Ig receptor-mediated cell surface stimuli.

The fact that those conditions optimal for secretory B-cell blockade failed almost completely to cause B memory cell blockade indicates that some signal from the thymic-dependent DNP_{22}-KLH is reaching at least some B cells. It is difficult to imagine that such near total escape from blockade could occur if blockade is routinely accomplished by a metabolically active but reversible switching event on B precursor cells. This argument seems especially cogent since memory cell formation was virtually complete when tested at 8 days post-blockade (Table VIII), but secretory cell detection was still minimal when primary DNP_{22}-KLH stimulation was given as late as 14 days after DNP_{59}-Ficoll injection (Fig. 2). At present it is not possible to tell whether the precursors for secretory and for memory B cells represent two distinct subpopulations in CBA/N mice, or whether there is actually only one targeted precursor population. If a single precursor population is involved, then the signal requirements for immunogen-triggered differentiation into memory cells must be minimal as compared to those required for maturation into immediate secretory function. In any case, memory cell formation in these B-cell defective mice must clearly escape blockade more readily than secretory cell activation.

The Significance of Hapten-Polysaccharide-Mediated Selective Immune Blockade. The present study emphasizes the importance of Ig receptors for induction of specific hapten-polysaccharide-mediated B-cell blockade in defective CBA/N mice. It has disclosed the refractoriness of B memory cells to blockade, and the inevitability of memory cell development despite the presence of profound secretory cell blockade. It also raises many questions among which are: (*a*) is blockade strictly and exclusively a B-cell function? (*b*) does blockade represent merely a passive saturation of surface Ig receptors on B cells? (*c*) is participation of a second category of non-Ig receptors necessary for transmission of the blockade signal? (*d*) is the signal for blockade on any given B cell reversible? (*e*) can memory cell formation proceed in the presence of a complete and sustained secretory B-cell blockade? and finally, (*f*) to what extent do the cellular and molecular events which lead to the X-linked blockade of CBA/N B cells resemble the events which cause hapten-specific blockade of normal B cells? Clear answers to any of the first five questions will be distinctly useful, but a definitive answer to the final question could ultimately be of major importance in efforts to control detrimental B-cell responses.

Summary

CBA/N mice, a mutant CBA subline, harbor an X-linked B-cell defect which prevents them from mounting immune responses to certain thymic-independent antigens such as pneumococcal polysaccharides and haptenated-Ficoll derivatives. These mice and the hybrid male progeny of CBA/N females are also exquisitely sensitive to a hapten-specific blockade of their otherwise adequate immune responses to thymic-dependent antigens such as *N*-2,4-dinitrophenylated-hemocyanin (DNP-KLH). As little as 10 ng of a DNP-Ficoll conjugate given 2 h before immunization with a 5,000-fold greater dosage of DNP-KLH, virtually abolishes the 4th-day direct plaque-forming cell (PFC) response specific for DNP. Responding hybrid (CBA/N × C3H/HeN) female mice are resistant to such blockade even at DNP-Ficoll dosages increased by three orders of magnitude. The DNP hapten and Ficoll must be chemically joined for this blocking effect to occur, and increasing the hapten derivatization of Ficoll increases its blockade-invoking capacity. Significant blockade can be produced by administering DNP-Ficoll as early as 4 days before or as late as 4 h after immunization with DNP-KLH. All currently available data point to the defective B cell as the target of this hapten-polysaccharide-mediated blockade. Mice bearing B memory cells, however, are refractory to such blockade. In addition, DNP-Ficoll injections which cause virtually total blockade of 4th-day primary direct PFC responses to DNP-KLH have little or no effect on the development of DNP-reactive B-cell memory measured at either 8 or 30 days. These findings suggest very different blockade susceptibilities for B cells or their precursors at various stages of differentiative development. Our findings also lead to the formulation of testable hypotheses regarding the mechanism of this selective B-cell blockade phenomenon.

We thank Barbara J. Duntley for her excellent technical assistance, and Dr. Phillip J. Baker for his helpful discussions and manuscript review.

Received for publication 28 February 1978.

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STREPTOCOCCAL M PROTEIN EXTRACTED BY NONIONIC DETERGENT

III. Correlation between Immunological Cross-Reactions and Structural Similarities with Implications for Antiphagocytosis*

BY VINCENT A. FISCHETTI‡

Despite antigenic diversity, group A streptococcal M protein has been able to maintain its biological property of being antiphagocytic. The mechanism by which this type-specific antiphagocytic effect is exerted at the molecular level has yet to be elucidated. Whereas immunological cross-reactions do occur between certain M types (1–3), rarely do these cross-reactive antibodies afford cross-protection (2, 4, 5). Therefore, we decided to examine the relationship of serological cross-reactions to structural relatedness between three streptococcal M types (M6, M12, and M14). By comparing the peptide maps between cross-reactive and non-cross-reactive M types, it was discovered that cross-reactions are correlated with the presence of several common peptides within these M proteins, pointing to some degree of structural similarity between these types. On the other hand, the non-cross-reactive M types yielded only three common peptides, of which only one was common to the three proteins analyzed. In addition, the data suggest that molecular conformation may play an important role in both immunological and biological activities of the M antigens. Mapping results also indicated that a major product of tryptic digestion found in all three M proteins examined was free lysine, suggesting a common lys-lys and/or arg-lys sequence.

Materials and Methods

Preparation of M Protein. Types 6, 12, and 14 M proteins were prepared as previously described (6) using nonionic detergent as the extracting agent.

Preparation of Tryptic Peptides. 300 µg (≅ 10 nmol) of M protein were dialyzed against 0.1 M ammonium bicarbonate buffer, pH 8, and then lyophilized. The protein was resuspended in 100 µl of 0.2 M ammonium bicarbonate, pH 8.0, and 3 µg of TPCK-trypsin (Worthington Biochemical Corp., Freehold, N. J.) was added in a total of 3 µl. The solution was allowed to incubate at 37°C for 7 h, and it was then lyophilized, resuspended in 0.2 ml H_2O, relyophilized, and stored desiccated until use.

Buffers. Sodium citrate buffers were utilized to elute the peptides from the sulfonated polystyrene column. Sodium citrate buffers of 0.2 M, pH 3.1; 0.5 M, pH 4.0; 1.0 M, pH 5; and 1.5 M, pH 6, were prepared in fresh double-distilled water with the molarities based on the sodium ion concentration. The pH was adjusted with hydrochloric acid which was redistilled over sodium dichromate (7) to remove contaminating amines. All buffers contained 0.2% nonionic detergent

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* Supported by grants AI 11822 and HL 03919 from the U. S. Public Health Service.

‡ Recipient of a U.S. Public Health Service Research Career Development Award.

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(octa-ethyleneglycol-dodecyl-ether) and 0.025% Merthiolate, and they were filtered through a 1.2-μm pore size filter (Millipore Corp., Bedford, Mass.).

o-Phthalaldehyde reaction buffer was prepared by adding 100 mg of Floram (Durrum Instrument Corp., Sunnyvale, Calif.), dissolved in 1 ml 95% ethanol, to 1 liter 0.8 M borate buffer, pH 10.5, containing 2 ml 2-mercaptoethanol and 3 ml Brij-35.

To avoid contamination by amines from the air, all buffers were maintained under positive pressure with nitrogen which had been bubbled through concentrated H_2SO_4.

Analytical System and Operational Procedures. Sulfonated polystyrene ion exchange resin type PA35 (Beckman Instruments, Inc., Mountainside, N.J.) was packed under pressure into a 0.6 × 15-cm jacketed high-pressure column (Glenco Scientific, Inc., Houston, Tex.) with the temperature maintained at 50°C. The column was flushed with 5 ml 0.2 M NaOH and equilibrated with the 0.2 M citrate buffer, pH 3.1. The trypsinized protein sample, suspended in 0.5 ml of the pH 3.1 citrate buffer, was introduced into the column using an automatic sample loading device (Altex Scientific Inc., Berkeley, Calif.). The column was eluted with a linear gradient using four chambers of a nine-chambered Varigrad gradient maker (VirTis Co., Inc., Gardiner, N. Y.), with each chamber containing 75 g of one of the four citrate buffers in the order pH 3.1, pH 4.0, pH 5.0, and pH 6.0. The buffer gradient (≅300 ml) was delivered to the column utilizing a high-pressure Milton Roy pump (Laboratory Data Control Div. Milton Roy Co., Riviera Beach, Fla.) at a flow rate of 24 ml/h (150–180 lb/in^2 pressure).

The column was monitored using a Technicon bubble-separation Autoanalyzer system similar to the one described by Catravas (8) with extensive modifications. 20% of the column effluent was combined with two volumes of the *o*-phthalaldehyde buffer, and the mixture was passed through a 3-min delay mixing coil. The reaction mixture was de-bubbled and passed through a 30-cm cooling jacket maintained at 15°C (to avoid the evolution of small bubbles in the 10-μl micro flowcell) and read by a Fluorimeter (American Instrument Co., Silver Spring, Md.). The flowcell chamber was also maintained at 15°C using a constant-temperature circulating cooling bath. To increase the stability and the sensitivity of the instrument, the bulb housing of the Fluorimeter was adapted to accept an Oriel low pressure UV lamp C-13-61 (Oriel Corp. of America, Stamford, Conn.). The flowcell was flanked by a Corning 7-60 primary filter and a Wratten 2A secondary filter (American Instrument Co., Silver Spring, Md.). The photomultiplier outputs were connected to a Linear 2-pen recorder (Linear Instruments Corp., Irvine, Calif.) and an Autolab system AA integrator (Spectra-Physics Inc., Mountain View, Calif.).

After each run (12 h), the column was flushed with 0.2 N NaOH for 15 min, and reequilibrated with the 0.2 M pH 3.1 starting buffer. By this method, more than 80 runs could be performed before the resin had to be removed and reconditioned as described in the Beckman Technical Bulletin for the PA35 resin.

Analysis of Data. Elution times of each peak were monitored by the Autolab AA integrator. Peaks having elution times differing by ≦2.3 min (in a total run time of 12 h) were considered identical. This time is based on the standard deviation of peak elution times obtained upon repetitive protein and amino acid standards over a period of six mo.

Isolation and Analysis of Free Lysine Peak. The majority of the sample from the PA35 column (≅80%) was passed through a delay coil with a transit time identical to the analyzed portion of the stream. The sample was collected by a fraction collector connected by an event marker to the recorder allowing for fractions to be correlated with peptide peaks. Fractions corresponding to the lysine peak were desalted as described by Drèze et al. (9). After lyophilization, half the sample was acid-hydrolized before amino acid analysis, and half was analyzed directly on a Durrum D500 amino acid analyzer.

Control Proteins. Tryptic peptide maps of 10–50 nm of staphylococcal protein A (Pharmacia Fine Chemicals, Piscataway, N.J.) and hemoglobin S (kindly supplied by Dr. James Manning of The Rockefeller University) were prepared as described for the M protein.

Results

Comparison of Peptide Patterns between Immunologically Related and Unrelated M Types. Recent radioimmunoassay studies from this laboratory utilizing M6 protein as the radiolabeled antigen and unabsorbed rabbit antisera directed against heterologous M types revealed that several antisera exhibited

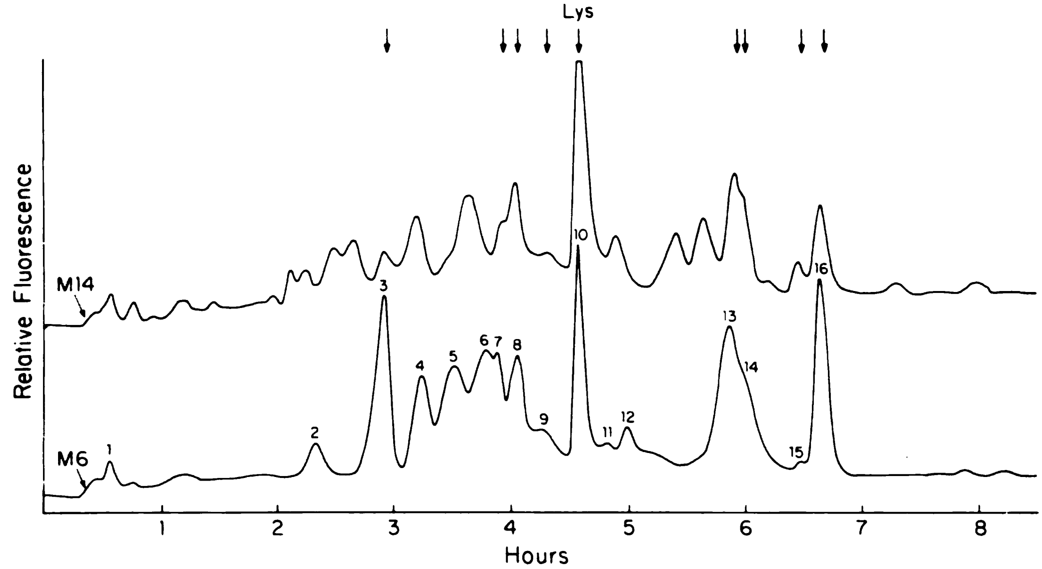

FIG. 1. Tryptic peptide map of M6 and M14 protein molecules chromatographed on Beckman PA35 resin and analyzed fluorometrically. Tryptic peptides of the M proteins (10 nm) were eluted from the resin by a linear gradient (0.2 M Na^+, pH 3.1 to 1.5 M Na^+, pH 6.0) and analyzed directly with *o*-phthalaldehyde. Integration and peak elution times were monitored by an Autolab AA integrator. Arrows signify those peaks that have identical elution times in both systems. Lys signifies the free lysine peak. No additional peaks were observed after 8 h in a total run of 12 h.

significant cross-reactions with the M6 antigen (10). The results suggested that these cross-reactive M types may have regions in common with the type 6 antigen, thus causing a certain degree of immunological cross-reaction with the M6 protein. In addition, competitive inhibition data indicated that unlike the M6 antisera which bound to the majority of the determinants on the M6 antigen, the cross-reactive antisera bound only to limited regions of the M6 antigen and were unable to neutralize the antiphagocytic effect of the M6 protein (10).

To determine the extent of structural similarity between cross-reactive and non-cross-reactive M types, as well as gaining insights as to the structural mechanisms involved in the common antiphagocytic effect of M protein, tryptic peptide maps of the 28,000–35,000 dalton molecules of two selected M proteins were compared to that of the M6 protein. M14 protein was chosen as a highly cross-reactive antigen since M14 antiserum at a 1:11 dilution bound up to 86% of the M6 antigen in a radioimmunoassay (10). On the other hand, M12 protein was selected as an essentially non-cross-reactive antigen since M12 antiserum bound only 15% (background levels) of the M6 antigen under the same conditions.

Fig. 1 reveals that tryptic peptide maps of the cross-reactive M6 and M14 proteins contain 9 of 16 peaks in common, suggesting a certain degree of structural relatedness between these two M types. Conversely, Fig. 2 illustrates that the M12 protein has only 4 peaks in common with the M6, indicating limited structural similarities between these two non cross-reactive M proteins. In addi-

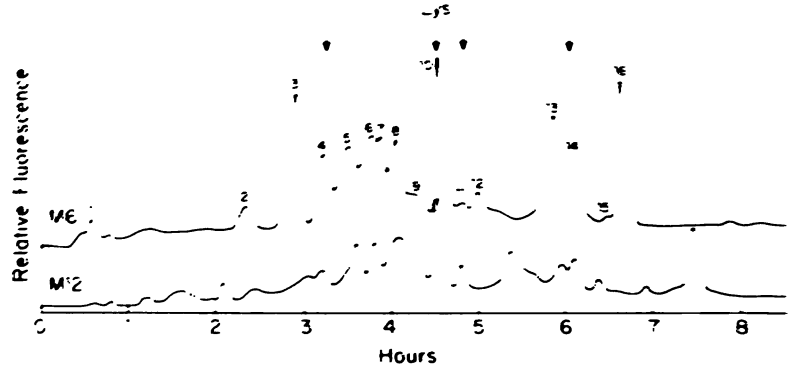

FIG. 2. Tryptic peptide map of M6 and M12 protein molecules. Same conditions as Fig. 1. Arrows indicate those peaks having an identical elution time in both systems. Lys signifies the free lysine peak.

tion, M6, M12, and M14 all release free lysine upon tryptic digestion (peak 10), suggesting that each contains lys-lys, or arg-lys sequences, or both. Surprisingly, the data also reveal that peptide 14 is the only peptide common to all three M types examined.

With relation to the free lysine peak, it was found that when the M protein chromatograms were compared with those of amino acid standards, no other peptide peak corresponded with any of the other standard amino acids. To further confirm the release of free lysine from a lys-lys sequence, tryptic peptides of hemoglobin (which has a known lys-lys sequence in both alpha and beta chains [11]) run on the peptide analyzer also exhibited a free lysine peak. In addition, amino acid analysis of the material in the lysine peak of the M protein digest verified the presence of only lysine. The quantity of lysine released by trypsin, however, was difficult to assess since tryptic digestion is not quantitative. However, it may be safe to assume that at least one lys-lys or arg-lys sequence is present in these three M protein molecules.

When peptide maps from different lots of the same M type were compared, reproducible patterns were observed, even down to the shoulders and shape of the individual peaks. Also, when tryptic peptide maps of control proteins such as staphylococcal protein A and hemoglobin were compared to those of the three M proteins, no similarity in peptide patterns was observed. Occasionally, however, a control peptide peak would overlap in elution time with a peptide in an M protein digest, suggesting that both peptides were of similar charge and/or sequence.

In the M protein chromatograms, no peptides were observed beyond 8 h of a 12-h run, and few peptides emerged before 2 h. Tryptic maps of the hemoglobin control, on the other hand, revealed several major peptides in these regions, suggesting that the peptides released by trypsin from the M-protein molecule are not generally of a highly or weakly charged nature.

Discussion

It is now well established that immunochemical cross-reactions between related proteins usually correspond directly with structural similarity (12). On the other hand, it has also been shown that the presence or absence of cross-reactions may be influenced by conformational changes within similar molecules (13); thus, cross-reactions between closely related proteins may not only be a consequence of sequence similarities. For instance, lysozyme and alpha lactalbumin, two proteins closely related in both primary structure (14) and three dimensional configuration (15, 16), exhibit no immunological cross-reactions (17). In spite of major primary sequence similarities between these two molecules, conformational configurations seem to be a major factor controlling the nonidentity of the antigenic determinants (17). Additionally, human, beef, lamb, goat, and sperm whale myoglobin molecules, having similar sequences and biological function, exhibit little or no immunological cross-reactions (13). Anomalies such as these could only be explained by inherent differences in conformation. For instance, it has been shown that antibodies raised against many types of native proteins seem to be directed against conformational structures rather than sequential determinants (18, 19).

In the case of the three M protein molecules examined in this report, it appears that the immunochemical cross-reactivity observed (10) may be influenced to some degree by the structural relatedness of the proteins involved. Despite the extent of cross-reactivity and structural similarity between M6 and M14 proteins, solid-phase radiocompetitive inhibition assays (10) demonstrated that M14 antiserum could bind to only 5% of the M6 determinants recognized by the M6 antiserum. This suggests that the conformational relatedness between these two M molecules with respect to the antigenic determinants available may actually be less than what peptide map analyses seem to indicate.

In our attempt to understand the mechanism by which the M protein molecule retains its antiphagocytic function while diverging antigenically, we argued that any minor sequence changes within the protein could promote changes in the immunological appearance of the molecule. However, these shifts would not cause major conformational alterations of the antiphagocytic portion of the protein. Major sequence changes, on the other hand, could eventually result in the disruption of the antiphagocytic function of the M-protein molecule. Hence, when these studies were initiated it was anticipated that the structural differences between M-protein types would be minor, with the changes being concerned primarily with those areas of the protein involved with its immunological reactivity (i.e., "constant" regions concerned with antiphagocytic determinants, and "variable" regions controlling the type specificity). It can be seen from these mapping results that even though all three M protein molecules have antiphagocytic determinants (evidenced by their ability to remove type-specific opsonic antibodies from serum), M6 and M12 have little structural relatedness. In other words, although M6 and M12 appear quite different both immunologically and structurally, their conformational appearance to an approaching phagocyte may be similar. These results suggest that biological activity may be based on conformational relatedness rather than primary sequence similarity (i.e., a tertiary structure exposing the same functional groups despite sequence variation).

This type of mechanism may have evolved by the action of selective pressure on the M-protein molecule. Without an alternate host, the group A streptococcus is faced with the dilemma of changing its immunological appearance while maintaining the ability to persist in man. Therefore, during evolutionary changes in the M antigen, possibly dictated by immunological pressure, those configurational alterations that accomplished both antigenic shifts without the loss of biological conformation could eventually survive.

The only consistency observed between all the maps in these studies is the release of free lysine by the action of trypsin and the presence of peptide 14. The former suggests the presence of either a lys-lys or arg-lys sequence, or both, within the protein. Since lysine is one of the major amino acids found generally in M protein by us (6) as well as others (20–22), this result is not unexpected. The presence of peptide 14 in all three M types, on the other hand, could suggest that this sequence may be involved in the antiphagocytic effect of the M molecules. However, no general opsonic antibody has ever been demonstrated, even against a limited number of M types, and this argues against a common antigenic sequence having the antiphagocytic property. In this regard, we have clearly shown that only when antibodies are directed against the majority of the determinants on the M protein will it neutralize the antiphagocytic effects of the molecule. On the other hand, avid antibodies binding to a limited number of determinants on the protein do not affect its biological property (10). Based on this data, it seems likely that through conformational shifts, the proximity of certain amino acids creates the required antiphagocytic environment for the M molecule, and that the spatial array of the determinants creating this environment may be such that several antibody molecules are necessary for its neutralization (10). An alternate hypothesis would be the presence of an antiphagocytic sequence which does not stimulate the production of antibodies by virtue of its similarity to a mammalian sequence. In this case, antibodies developed against the antigenic domains adjacent to this sequence would sterically neutralize the antiphagocytic effect of the active region. Sequence and immunochemical data are presently being accumulated in an attempt to answer these questions more conclusively.

Summary

Three immunologically cross-reactive and non-cross-reactive streptococcal M proteins were analyzed by a chromatographic tryptic peptide mapping system. The results indicate that cross-reactions correlate with the extent of structural similarity among the M protein molecules analyzed. The data also reveal that free lysine is released by the action of trypsin from these three M proteins, suggesting a common lys-lys or arg-lys sequence. In addition, only one peptide has been found to be common within all three M types. This limited structural relatedness among the three M proteins examined indicates that sequence variation plays a major role in the immunological specificity of the M antigens. However, despite sequence variation, all M protein molecules have a common antiphagocytic activity. The fact that no common opsonic antibody has yet been found, even against limited M types, argues against this biological activity being solely the result of a common sequence. Based on these data, it is suggested that the antiphagocytic effect of M protein may be due to a conformationally created environment on the surface of the molecule which is selected by both immunological and biological pressure.

The author wishes to thank Doctors E. C. Gotschlich, J. B. Zabriskie, Maclyn McCarty, I. van de Rijn, and J. W. Tauber, and Ms. E. De Falco for their suggestions in the preparation of this manuscript. I also wish to acknowledge Ms. Nancy Fertitta for her superb technical assistance in these experiments, and Ms. Sheenah McManus for her excellent assistance in the isolation and analysis of the amino acids.

Received for publication 28 December 1977.

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SYNTHESIS OF FIBRONECTIN BY CULTURED HUMAN ENDOTHELIAL CELLS*

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Plasma fibronectin (FN)[1] is probably the major nonimmune particulate opsonin of blood (1, 2). FN is also cross-linked to fibrin during the final stage of blood coagulation (3, 4). It was first identified in 1948 and was previously referred to as cold insoluble globulin (5). FN is a glycoprotein composed of high molecular weight polypeptide subunits which is present in human plasma in concentrations of 300–400 μg/ml (3, 6). In the last several years, an immunologically similar protein with a 200–200,000 mol wt polypeptide subunit has been found in the postculture medium of fibroblasts; the same antigen has been identified by immunofluorescence microscopy in vertebrate basement membrane and loose connective tissue (7–9). This antigen is also known as surface fibroblast antigen (7), large, external, transformation-sensitive protein (10), and cell-surface protein (11). Cultured fibroblasts (12–15) and astroglial cells (16) have previously been identified as sites of syntheis of FN. FN in fibroblast cultures is found both in a soluble form in conditioned medium and in insoluble forms in the extracellular connective tissue matrix and on the cell surface. The insoluble forms may be spatially related to collagen for when fibroblast cultures are studied by immunofluorescence microscopy, the same filamentous extracellular structures are labeled by both FN and collagen antibodies (17). Also, FN has recently been shown to bind to collagen (18). FN is probably the protein in serum-containing tissue culture medium which mediates initial cell attachment and spreading on the surfaces of culture vessels (19–21). FN synthesized and deposited underneath cells may mediate adhesion of cells to culture dishes and other, more biologically relevant surfaces (22). Transformed cultured fibroblasts generally lack cell surface FN (10, 11, 14–16, reviewed in 23). When purified FN is added to transformed cells, the transformed cells assume a more normal morphology and state of adhesiveness (24, 25).

We and others have previously shown that cultured human endothelial cells synthesize extracellular material which is morphologically and immunologically like amorphous basement and contains basement membrane collagen (26, 27). Cultured endothelial cells also appear to synthesize extracellular material morphologically similar to microfibrils and elastic fibers (26). Recent immunofluorescence microscopic studies have demonstrated FN in the endothelium and subendothelium of vessels in developing chicks (6) and in human tissues (28, 29).

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* Supported by grants from the National Institutes of Health, HL-18828, the Arnold R. Krakower Hematology Foundation, and the University of Wisconsin Graduate School.

‡ Recipient of National Institutes of Health Research Career Development Award (1 K04 HL 00237) and a Career Scientist Award from the Irma T. Hirschl Trust.

[1] Abbreviations used in this paper: FN, fibronectin; MEM, minimal essential medium; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline.

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In this paper, we report that cultured human endothelial cells synthesize FN with a polypeptide subunit mol wt of 200,000. Cultured endothelial cells secrete FN into culture medium and incorporate FN into the extracellular matrix. We suggest that endothelial cells may be a major site of synthesis of FN.

Materials and Methods

Cell Culture Techniques and Culture Media. Human endothelial cells were derived from umbilical cords and cultured by using methods and materials previously described (30). Endothelial cells were cultured in plastic T-25 or T-75 flasks (Corning Glass Works, Science Products Div., Corning, N. Y.). The flasks were pretreated by incubation overnight at 4°C with 0.2% gelatin (Difco Laboratories, Detroit, Mich.) in water. The gelatin was removed just before use. Coverslips on which cells were cultured for immunofluorescence microscopy were similarly treated with gelatin. The culture medium for endothelial cells consisted of medium 199 (Flow Laboratories, Inc., Rockville, Md.) containing either 20% fetal calf serum (Reheis Chemical Co., Chicago, Ill.) or 20% normal rabbit serum (Pel-Freez Farms Inc., Rogers, Ark.). The culture media also contained penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM) (all Flow Laboratories) and Hepes buffer (15 mM, pH 7.4, Sigma Chemical Co., St. Louis, Mo.).

Immunofluorescence Microscopy. 2–7 days after subculture, cultured cells were prepared and processed for immunofluorescence microscopy by previously described methods (30, 31). The goat anti-rabbit IgG which was purified by affinity chromatography and conjugated with fluorescein according to methods previously described (32) was kindly provided by Dr. Carl G. Becker (Cornell University Medical College, New York). The various rabbit antibodies and normal rabbit serum were used at dilutions of 1:10 or 1:20 and 20 μl of antiserum were used per coverslip. Immunofluorescence microscopy and photomicrography were performed by methods previously described (30).

Antibodies and Antigens. Plasma FN and goat and rabbit antibedies to human plasma FN (designed anti-FN) were prepared as previously described (3, 4). The unabsorbed rabbit anti-FN had no detectable anti-factor VIII procoagulant activity or anti-von Willebrand factor activity when assayed by methods previously described (33, 34). However, due to the possibility of small amounts of contaminating factor VIII antigen in the material used to elicit the anti-FN sera, all the anti-FN sera used in this study were absorbed with purified plasma factor VIII (35) before use. Anti-FN sera were absorbed by reacting 4 vol of anti-FN with 1 vol of purified factor VIII (0.98 mg/ml) at 37°C for 1 h and overnight at 4°C. The anti-FN sera were then centrifuged at 8,000 *g* for 20 min at 4°C. When the absorbed anti-FN sera and a well characterized anti-factor VIII serum (35) were reacted in immunodiffusion against a mixture containing FN and factor VIII, lines of nonidentity were seen and the anti-factor VIII also detected free factor VIII antigen in the anti-FN sera. When anti-factor VIII and absorbed anti-FN sera were tested by immunofluorescence microscopy with confluent cultured endothelial cells, the antisera gave different patterns of fluorescence. The rabbit anti-FN stained mainly an extensive meshwork of extracellular fibrils while the anti-factor VIII stained only intracellular granules.

The antisera to human serum albumin was obtained from Behring Diagnostics, American Hoechst Corp., Somerville, N. J. The antisera to ovalbumin was kindly provided by Dr. Gordon D. Ross, Cornell University Medical College, New York. The antisera to anti-thrombin III and prothrombin were provided by Dr. Robert Rosenberg, Harvard Medical School, Boston, Mass. All antisera were absorbed with aluminum hydroxide and heat-inactivated before use.

Quantitative Absorption Studies. Rabbit anti-FN was diluted 1:4 with phosphate-buffered saline ([PBS] 0.145 M NaCl, 0.01 M phosphate, pH 7.4) and 0.2-ml portions pipetted into a series of Eppendorff centrifuge tubes. To these were added serial twofold dilutions (0.2 ml) of purified human FN, the highest concentration being 488 μg/tube. The tubes were incubated with frequent shaking at 37°C for 2 h and then at 4°C for 72 h. The precipitate harvested by centrifugation was washed four times in PBS, dissolved in 1 N NaOH, and protein content determined by the Lowry et al. procedure (36). Portions of each supernate were used to treat cultured endothelial cells as described above to determine if absorption of anti-FN serum at equivalence with purified FN would inhibit immunofluorescence staining. These preparations were compared with cultured endothelial cells treated with unabsorbed anti-FN at the same final dilution (1:20) as the supernate and with cells treated with pooled normal rabbit serum. Using the data obtained from the quantitative immunoprecipitation study (Fig. 2), we calculated that the anti-FN serum contained 2.46 mg/ml of specific anti-FN antibody.

Radial Immunodiffusion. Radial immunodiffusion was performed by the method of Mancini et al. (37) with goat anti-FN at a final dilution of 1:140. Confluent endothelial cells were cultured for 3 days in T-75 flasks in 10 ml of culture media containing 20% fetal calf serum. The postculture media was removed, centrifuged to remove debris, lyophilized, reconstituted in 1/5 the original volume of PBS, dialyzed against PBS, and analyzed. Human plasma of known FN concentration served as a secondary standard. The primary standard was purified plasma FN; protein concentration of the primary standard was determined by absorbance at 280 nm (6). The minimal level of FN detectable by this technique in a 10-μl sample was 40 ng. Thus, the minimal level detectable in the culture media was 8 μg/flask (10 ml of culture media). The goat anti-FN sera used in this study did not detect FN in preculture media or in the fetal calf serum used in the culture medium.

Immunodiffusion analysis. Immunodiffusion analysis was performed on glass slides containing 1% agarose in 50 mM Tris-barbital buffer pH 8.2. Samples to be tested (10 μl) were placed in 4-mm diameter wells and the plates incubated at room temperature for 48 h. The anti-FN was used at a dilution of 1:2. The plates were washed extensively with Tris-buffered saline and then water, stained with Coomassie Brilliant Blue, and photographed.

Radioactively Labeled FN Synthesized by Endothelial Cells. Radioactively labeled FN synthesized by endothelial cells was prepared by culturing monolayers of human endothelial cells in T-75 flasks in medium 199 containing 20% normal rabbit serum until the cells were confluent. The medium was then removed and the cells washed three times with Dulbecco's PBS. The cells were incubated in leucine-free minimal essential medium (MEM) (Flow Laboratories) containing 20% heat inactivated rabbit serum for 30 min at 37°C. This medium was removed and replaced with 10 ml of fresh leucine-free MEM containing 20% heat inactivated rabbit serum, L-[4, 5-^{3}H] leucine (20 μCi/mol, sp act 50 Ci/mmol, Amersham Corp., Arlington Heights, Ill.), and Trasylol (50 U/ml, FBA Pharmaceuticals, Inc., New York) and the cells incubated for 24 h at 37°C. The postculture medium was removed and the following inhibitors added to it to yield the listed final concentration: 0.4 mM phenyl methyl sulfonyl fluoride (Sigma Chemical Co.), 5 mM *N*-ethyl maleimide (Sigma Chemical Co.), 10 mM EDTA, and 1 μM pepstatin (Protein Research Foundation, Osaka, Japan). The postculture medium was centrifuged at 40,000 *g* for 1 h at 4°C to sediment particulate debris. The supernate was made up to 50% saturation with ammonium sulfate, incubated at 4°C overnight, and centrifuged at 40,000 *g* for 1 h at 4°C. The precipitate from 2 T-75 flasks was redissolved in PBS (1/10 original volume) containing the four inhibitors listed above, dialyzed at 4°C against two changes of 2 liters each of PBS, and stored frozen at −40°C. This procedure yielded approximately 2 ml of a protein solution containing 18.6 mg/ml protein and 4.54×10^7 dpm/ml (sp act 2.44×10^6 dpm/mg).

The [^{3}H]-labeled endothelial cell monolayers were washed four times with medium 199 containing 10^{-4} M leucine and once with medium 199 containing 10^{-4} M leucine and 0.4 mM phenyl methyl sulfonyl fluoride. The cell monolayers were then extracted for 2 h at 37°C with medium 199 containing 1 M urea and 0.4 mM phenyl methyl sulfonyl fluoride (11, 38). The extract was removed and *N*-ethyl maleimide, EDTA, and pepstatin added to yield the same final concentrations as used in processing the postculture medium. The urea extract was centrifuged at 40,000 *g* for 15 min and then dialyzed against PBS at 4°C. This procedure yielded approximately 6.5 ml of a solution containing 1.75×10^6 dpm/ml.

Immunodiffusion Analysis. Immunodiffusion analysis was performed on glass slides contain-by cultured endothelial cells was isolated from fractions derived from postculture medium and from urea extracts of cellular monolayers by a method using anti-FN. The method used a Protein A-Sepharose column and is a modification of the method of Kessler (39). To inhibit proteolytic enzymes, phenyl methyl sulfonyl fluoride (0.4 mM), EDTA (10 mM), *N*-ethyl maleimide (5 mM), and pepstatin (1 μM) were added to all buffer and protein solutions used in these procedures.

Protein A-Sepharose 4B (Protein A bound to Sepharose 4B, Pharmacia Fine Chemicals, Piscataway, N. J.) was washed twice with 0.15 M NaCl, 0.02 M Tris-HCl, pH 7.4 (TBS-Tris-buffered saline). 0.2 ml of the washed beads, 0.2 ml TBS, and 0.15 ml of the anti-FN were incubated with end-over-end rotation for 2 h at 20°C. To saturate the remaining Protein A binding sites, 2 ml of heat-inactivated normal rabbit serum was added, the mixture incubated for 1 h at

4°C, and the beads then washed six times with PBS. The washed beads were suspended in 0.75 ml of PBS, 0.25 ml of the [^{3}H]-labeled ammonium sulfate precipitated postculture meidum or 0.9 ml of the dialyzed cell extract was added, and the mixture incubated overnight at 4°C. The suspension was transferred to a 0.6 × 15 cm chromatographic column (Glenco Scientific Inc., Houston, Tex.) and the excess fluid drained off. The beads (0.2 ml packed volume) were washed sequentially with PBS (6 ml), 5 M KI (8 ml), 1% Triton X-100 (Sigma) in PBS (8 ml), and PBS (5 ml). The beads were then eluted with 1 M acetic acid and the eluate analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The washes with 5 M KI and 1% Triton X-100 were added because in preliminary experiments FN was noted to stick nonspecifically to Protein A-Sepharose. Kessler (39) has shown that 5 M KI and the nonionic detergent NP-40 do not release appreciable amounts of antigen-antibody complexes bound to Protein A. We found that washes with 5 M KI and 1% Triton X-100 in PBS release nonspecifically bound FN but did not release FN bound to anti-FN. Control experiments were performed with anti-ovalbumin and anti-human serum albumin.

Immunoisolation of [^{3}H] Endothelial Cell FN by Double Antibody Immunoprecipitation. Double antibody immunoprecipitation reactions were carried out on [^{3}H]leucine-labeled ammonium sulfate precipitated endothelial cell postculture medium prepared as above except that the endothelial cells were labeled while in fetal calf serum. The cells were thus cultured to avoid adding large amounts of rabbit gamma globulins which would have interfered with the second immunoprecipitation step (goat anti-rabbit IgG precipitating the rabbit anti-FN). 38 μl of rabbit anti-FN was added to 0.5 ml [^{3}H]-labeled ammonium sulfate precipitated postculture medium and the mixture incubated for 2 h at 37°C and overnight at 4°C. Goat anti-rabbit IgG (0.2 ml) was added and the mixture further incubated for 2 h at 37°C and for 2 days at 4°C. The mixture was centrifuged at 8000 *g* for 5 min at 4°C. The supernate was removed and saved for analysis and the precipitate washed six times by centrifugation with PBS containing 0.5% Triton X-100 (39). The supernates and pellets were then analyzed by SDS-polyacrylamide gel electrophoresis. Control experiments were performed with anti-ovalbumin and anti-human serum albumin. Preliminary quantitative immunoprecipitation experiments were performed to determine the equivalence point for the reaction between the goat anti-rabbit IgG and the various antibodies.

SDS-Polyacrylamide Gel Electrophoresis. SDS-polyacrylamide gel electrophoresis was performed in 3% acrylamide-0.5% agarose gels by the method of Weinstein et al. (40). Samples in 1 M acetic acid were prepared for electrophoresis by heating in sample buffer for 1 h at 37°C. The samples were then dialyzed against running buffer containing 1% SDS, 0.4 mM phenyl methyl sulfonyl fluoride, 5 mM *N*-ethyl maleimide, 10 mM EDTA, and 1 μM pepstatin for 2 h at 20°C. Samples to be reduced were treated with 3 μl/100 μl of a 1.4 M dithiothreitol solution by boiling for 5 min. Immunoprecipitates and samples not in acetic acid were solubilized by boiling for 5 min in sample buffer. Gels were sectioned into 2-mm thick slices and processed for liquid scintillation counting either as previously described (35) or by incubating the gel slices in 10 ml of 3% Protosol in Econofluor (New England Nuclear Corp., Boston, Mass.) for 16–24 h at 37°C (the two methods yielded the same results). The samples were counted in a Searle Mark III liquid scintillation counter (Searle Analytic Inc., Arlington Heights, Ill.). Purified human plasma factor VIII (subunit mol wt 202,000) used as a marker was prepared as previously described (35). Purified plasma FN used as a marker (subunit mol wt 200,000) was purified as previously described (3).

The 3% polyacrylamide-0.5% agarose system was chosen because in this system when samples are run unreduced, factor VIII and plasma FN are completely separated from each other whereas with reduction they have the same mobility (40).

Results

Immunofluorescence Studies. When coverslips of confluent cultures of human endothelial cells, cultured in normal rabbit serum and fixed in acetone, were sequentially incubated with rabbit anti-FN and fluorescein-conjugated goat anti-rabbit IgG, meshworks of extracellular fibrils covering all the cells were brightly stained (Fig. 1). When subconfluent endothelial cells were similarly stained, few immunofluorescent extracellular fibrils were seen though large numbers of brightly fluorescent intracellular granules were present. As

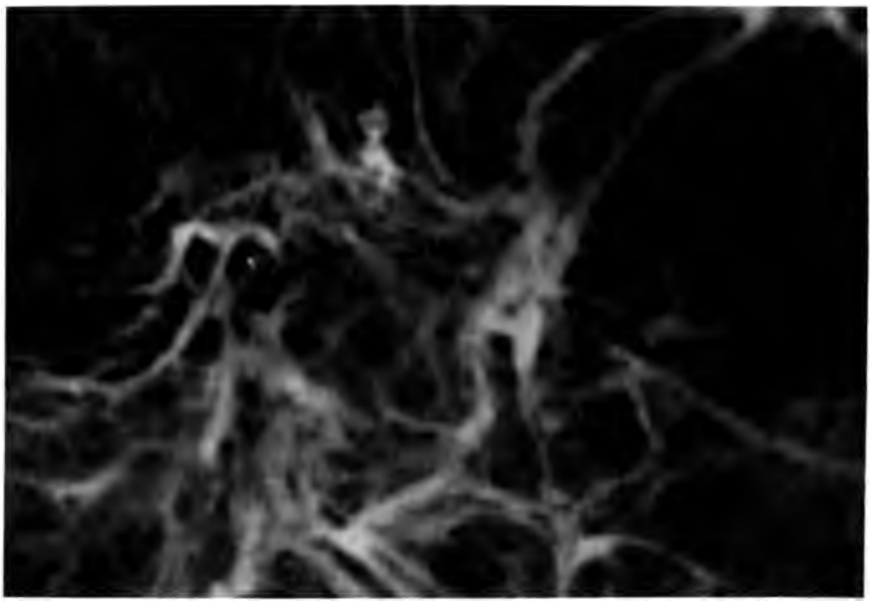

FIG. 1. Immunofluorescence study of cultured human endothelial cells cultured in rabbit serum. Cells were treated with rabbit anti-FN and then with fluorescein-conjugated goat anti-rabbit IgG. A meshwork of extracellular fibrils is brightly stained; the cells are very lightly stained and are barely visible because the extracellular matrix stains so brightly (× 960).

the cells became more confluent, the numbers of intracellular immunofluorescent granules decreased somewhat and the extent of the extracellular immunofluorescent fibrillar meshwork greatly increased. The fibrils were not related to areas of cell-cell junctions, instead they appeared to form a continuous meshwork underlying the cells. No staining was seen when the cultured endothelial cells were stained with normal rabbit serum, anti-prothrombin, or antisera to anti-thrombin III instead of anti-FN. To demonstrate the endothelial origin and homogeneity of the cells, parallel coverslips were also stained for factor VIII antigen using an antibody to human factor VIII antigen (31, 35). All the cells were brightly stained thus confirming their endothelial origin (31).

Immunofluorescence staining of endothelial cells was completely inhibited by prior absorption at equivalence of anti-FN serum by purified FN (Fig. 2). Supernates from the zone of antigen excess did not stain cultured endothelial cells, whereas supernates in the zone of antibody excess did.

Endothelial Cell FN. For this study, endothelial cells were cultured in media containing 20% fetal calf serum since the goat anti-FN was unable to detect FN in fetal calf serum by immunodiffusion analysis. Pre- and postculture media were concentrated five-fold and assayed for FN by radial immunodiffusion using goat anti-FN. Concentrated preculture media contained no detectable FN (Table I). However, concentrated postculture media from four different endothelial cell strains did contain significant amounts of FN.

Immunodiffusion Studies. On immunodiffusion analysis, goat anti-FN reacted with a line of identity when tested against endothelial cell postculture

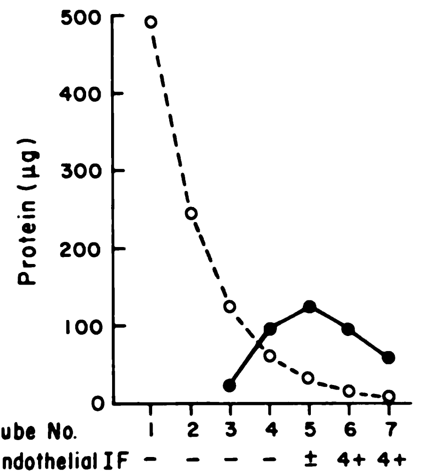

FIG. 2. Quantitative precipitin analysis of the FN-anti-FN reaction. Serial dilutions of purified human FN (-O-) were added to a constant amount of rabbit antiserum to FN. The resulting immunoprecipitates (-●-) were quantitated by the Lowry procedure. The ability of the supernates to stain cultured endothelial cells was tested by immunofluorescence as detailed in the Materials and Methods section.

TABLE I
FN Content of Pre and Postculture Media

Material tested*	FN‡
	µg/culture flask
Preculture media	<8
Postculture media	
Culture	
1	50
2	25
3	55
4	77

* Four different endothelial cell lines were cultured for 3 days and the media were concentrated fivefold and analyzed by radial immunodiffusion.

‡ The minimal amount of FN detectable by this technique was 8 µg/culture flask. The values represent averages of two sets of analyses on multiple dilutions of each sample.

medium and human plasma (Fig. 3). Preculture medium, which contained fetal calf serum, did not form a precipitin line when tested against the goat anti-FN serum.

Immunoisolation of Endothelial Cell FN. FN synthesized by endothelial cells was isolated from fractions of postculture media derived from endothelial cells cultured in 20% normal rabbit or fetal calf serum with [^{3}H] leucine in the presence of Trasylol (50 U/ml). The [^{3}H] FN was isolated either by a technique using Protein A-Sepharose or by double antibody immunoprecipitation. The Protein A-Sepharose technique utilized a rabbit anti-FN sera and cells cultured and labeled in rabbit serum to avoid interspecies cross-reactivity. The isolated

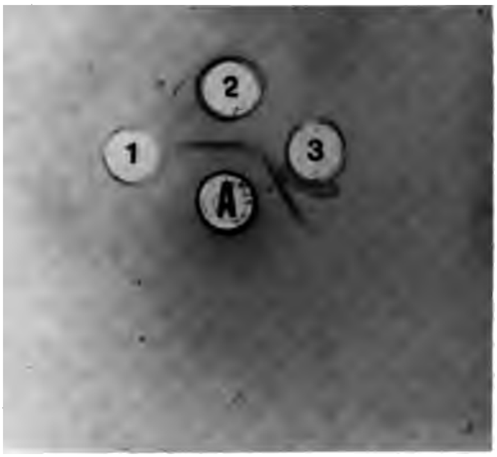

FIG. 3. Immunodiffusion analysis of FN synthesized by endothelial cells. The center well (A) contained goat anti-human FN. The numbered wells contained: 1) preculture medium; 2) human plasma; and 3) postculture medium. The stained immunodiffusion plate shows a line of identity between the FN in human plasma and the FN present in endothelial cell postculture medium. No FN was detected in the preculture medium.

[^{3}H] FN was characterized by SDS-polyacrylamide gel electrophoresis with and without reduction by dithiothreitol.

A fraction obtained from [^{3}H] leucine-labeled endothelial cell postculture medium by precipitation with ammonium sulfate at 50% saturation was electrophoresed, sliced, and counted for radioactivity (Fig. 4A). This gel contained three major radioactive peaks, one of which comigrated with the human plasma FN used as a marker. The radioactively labeled ammonium sulfate precipitated postculture medium was subjected to immunoisolation using anti-FN coupled to Protein A-Sepharose. After this procedure, analysis of the depleted labeled postculture media by SDS-polyacrylamide electrophoresis revealed that the radioactive peak comigrating with the plasma FN marker (Fig. 4A) had almost completely disappeared (data not shown). SDS-gel electrophoresis (after reduction) of the acetic acid eluate from the anti-FN Protein A-Sepharose column (Fig. 4B), revealed one radioactive band with a mol wt of 200,000 which comigrated with the plasma FN marker. When the acetic acid eluate was electrophoresed without reduction (Fig. 4C), one radioactive band was seen and it comigrated with the unreduced plasma FN marker. Control studies performed with anti-human serum albumin and anti-ovalbumin instead of anti-FN did not isolate any labeled material (Figs. 4B and 4C). In this gel system (3% acrylamide-0.5% agarose) after reduction, the plasma factor VIII marker comigrated with the plasma FN marker. However, when factor VIII was electrophoresed without reduction, it did not enter the gel while the plasma FN marker did. Thus, this system could be used to completely separate FN and factor VIII (40).

Labeling patterns identical to those seen in Fig. 4 were seen when radioactively labeled ammonium sulfate precipitated postculture medium was subjected to double antibody immunoprecipitation with rabbit anti-FN and goat anti-rabbit IgG. Control double antibody immunoprecipitation experiments

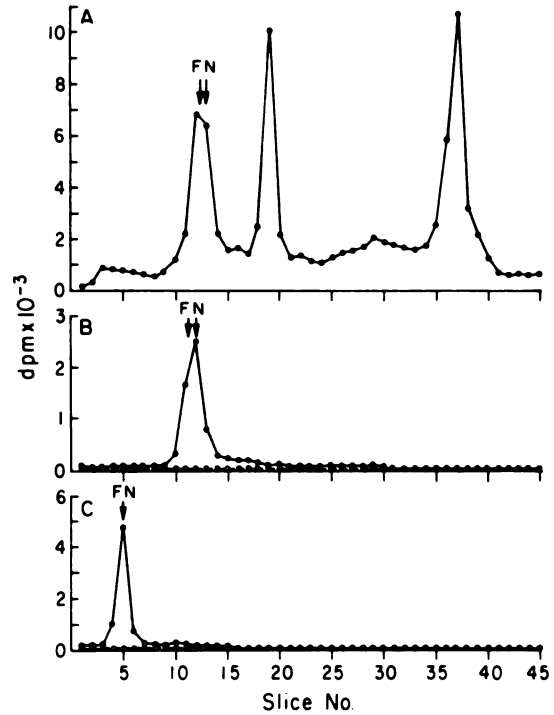

FIG. 4. Protein A-Sepharose isolation of FN synthesized by cultured endothelial cells. Distribution of radioactivity in SDS-polyacrylamide gels of: (A) fraction derived from [^{3}H] leucine-labeled endothelial cell postculture medium by precipitation with 50% saturated ammonium sulfate (reduced with dithiothreitol); (B) fraction derived by acetic acid elution of an anti-FN Protein A-Sepharose column which has been reacted with the [^{3}H] leucine labeled, ammonium sulfate precipitated endothelial cell postculture medium shown in (A) and then washed as detailed in the Materials and Methods Section (-●-●-). Control experiments were performed by using anti-human serum albumin and anti-ovalbumin (-O-O-) instead of anti-FN (all reduced with dithiothreitol); (C) same as (B) except unreduced by dithiothreitol. The arrows labeled FN represent the location of the plasma FN marker run on companion gels. When reduced, plasma FN electrophoreses as a closely spaced doublet with an average mol wt of 200,000, hence the double arrow.

using anti-human serum albumin and anti-ovalbumin instead of anti-FN precipitated no labeled material.

Since essentially all (≥95%) the radioactivity in the high molecular weight peak comigrating with plasma FN marker (Fig. 4A) was FN and that peak represented ~15% of the output of [^{3}H] leucine-labeled protein by the endothelial cells, we estimate that FN represents ~15% of the protein released by endothelial cells into the culture medium.

Endothelial cell monolayers labeled with [^{3}H] leucine were extracted with 1 M urea for 2 h at 37°C since it has been shown that insoluble fibroblast FN can be extracted using this procedure (11, 38). The urea extract was analyzed by the Protein A-Sepharose technique described above. Labeling patterns identical to those seen in Fig. 4B and 4C were obtained. Control experiments performed with anti-ovalbumin instead of anti-FN did not isolate any labeled material.

Discussion

The studies reported here demonstrate that cultured human endothelial cells synthesize and release FN into culture media and incorporate FN into the extracellular matrix. The radioactive FN secreted by endothelial cells into the culture medium and incorporated into the extracellular matrix comigrated with the plasma FN marker on SDS-polyacrylamide gel electrophoresis both with and without reduction and had a single polypeptide subunit of 200,000 mol wt similar to the plasma FN marker. On immunodiffusion analysis, the FN secreted by cultured endothelial cells into their postculture medium formed a line of identity with human plasma when both were reacted against anti-FN. Thus, the FN synthesized and secreted by endothelial cells and plasma FN are immunologically identical and are both disulfide bonded dimers with subunits of the same molecular weight.

The validity of our studies is dependent on the specificity of the anti-FN sera used. These antibodies have previously been shown to form a single line when tested against human plasma by immunodiffusion analysis and immunoelectrophoresis (3, 4). The quantitive immunoprecipitin curve and the loss of immunofluorescence at equivalence (Fig. 2) also argue strongly for monospecificity. We have previously shown that cultured endothelial cells contain, synthesize, and release factor VIII antigen (31,33, 35). To avoid the possibility of contamination of the anti-FN with trace amounts of anti-factor VIII, all anti-FN sera were absorbed with purified plasma factor VIII before use. These absorptions and the patterns of mobility of the [^{3}H] FN peaks seen in Fig. 4 rule out the possibility that the isolated radioactive peaks seen in Figs. 4B and 4C are factor VIII antigen.

FN is one of the major proteins synthesized and secreted by cultured endothelial cells. It appears to constitute ~15% of the protein released by the cells into the culture media. By comparison, factor VIII antigen represented only 5.5% of a high molecular fraction (separated by Sepharose 6B) prepared from the same starting material (31) and thus probably represented <1% of the total protein released by the cells into the culture medium. These estimates are consistent with the concentrations of these proteins in plasma, i.e. factor VIII antigen ~5–10 μg/ml and plasma FN ~330 μg/ml (1). Cultures of human endothelial cells secreted (Table I) an average of 51.8 μg FN/25 cm^2 dish during 3 days in culture. By comparison, a variety of first passage human embryonic cell strains secreted an average of 106 μg FN/25 cm^2 dish (equivalent) during 3 days in culture (15, 41). Thus, endothelial cells synthesize and release into their culture medium significant amounts of FN having subunits of the same molecular weight as those found in human plasma.

FN has been shown to be present in the endothelium and subendothelium of a variety of blood vessels. Linder et al. (7) in studies on the developing chick embryo showed that in addition to primitive mesenchymal tissue and basal lamina, endothelial cells and the subendotheium of arteries and veins and also of brain capillaries contained FN when tissue sections were studied by immunofluorescence microscopy. FN was absent in parenchymal cells in a variety of organs such as the liver, striated and smooth muscle, brain (except neural sheaths), cartilage, and bone. Similar immunofluorescence studies on a variety of human tissues have shown that FN is most prominently seen associated with endothelial cells (28, 29). In addition, FN was also seen in basal lamina, the media of blood vessels, and loose connective tissue (29). Our present data in conjunction with these immunofluorescence studies and the location of endothelial cells (adjacent to the blood stream) suggest that endothelial cells are a major site of synthesis of plasma FN.

A physiologic role of FN in plasma has just been demonstrated. FN has shown to be identical to human α_2 opsonic glycoprotein which is thought to have the important restorative function of mediating the uptake of colloids and intravascular particulates by the reticuloendothelial system (1, 2, 42, 43). Our in vitro study and the immunofluorescence studies performed on tissue sections by others (7, 28, 29) have shown that the extracellular matrix of endothelial cells contains FN. The physiologic role of FN in tissues has been inferred from its effects on cultured cells. FN is necessary for the attachment of tissue culture cells to culture vessels (19–22). FN also increases the strength of attachment of transformed cells to culture vessels and thus changes their morphology (though not their growth characteristics and malignant potential) (24, 25). The mechanism of these two effects is unknown. Fibroblast cultures contain fibrils which stain for both FN and collagen when examined by immunofluorescence microscopy (17). The basis for this congruence of location may be the recently described noncovalent interactions of FN with other FN molecules (44) and with collagen (18). FN crosslinks with itself and other proteins through disulfide links (9, 45). FN on cell surfaces is known to be immobile and may serve as an anchorage point for cell attachment (44). Thus, FN in the subendothelium may be an important structural component which plays a role in endothelial cell attachment and adhesiveness under normal conditions and after injury to the vascular wall.

Summary

Plasma fibronectin is probably the major nonimmune particulate opsonin in blood and is cross-linked to fibrin during the final stage of blood coagulation. Fibronectin also occurs in an insoluble form in basement membranes especially those underlying endothelial cells and in loose connective tissue.

Fibronectin was demonstrated in cultured human endothelial cells and in the surrounding extracellular matrix by immunofluorescence microscopy by using antibody to human plasma fibronectin. Cultured human endothelial cells released fibronectin into the culture medium which was immunologically identical to the fibronectin in human plasma. Cultured human endothelial cells were labeled with [^{3}H] leucine. The radioactive fibronectin present in the endothelial postculture medium and in urea extracts of cellular monolayers was isolated with either anti-fibronectin coupled to Protein A-Sepharose or double antibody immunoprecipitation and characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. When reduced, the [^{3}H] fibronectin synthesized by cultured endothelial cells had the same mol wt (~200,000) as plasma fibronectin. Unreduced, the [^{3}H] fibronectin synthesized by endothelial cells migrated as a dimer, as did plasma fibronectin. Fibronectin accounted for ~15% of the protein synthesized and released by endothelial cells into the culture medium.

Thus, cultured endothelial cells synthesize fibronectin, secrete it into the culture medium, and incorporate it into extracellular matrix. The results suggest that the endothelial cell is potentially a major site of synthesis of circulating plasma fibronectin. In addition, fibronectin derived from endothelial cells may be an important structural component of the subendothelium.

The authors wish to thank Lilane Bronson, Christine Baranowski, and Louise Wolf for their excellent technical assistance.

Received for publication 23 February 1978.

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INTERACTION OF β1H GLOBULIN WITH CELL-BOUND C3b: Quantitative Analysis of Binding and Influence of Alternative Pathway Components on Binding*

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Participation of C3b, the major cleavage product of C3, in both the classical and alternative pathways of complement activation is modulated by several control proteins (1–3). Two of these, C3b inactivator (C3bINA)[1] (4, 5) and β1H-globulin (β1H) (2), have been extensively purified and characterized. It is now apparent that C3bINA is a protease, and that it blocks the biologic activities of C3b by cleaving peptide bonds in this molecule (1, 4, 6). The second protein, β1H, potentiates the activity of C3bINA; indeed, recent evidence indicates an absolute requirement for β1H in the cleavage of fluid phase C3b by C3bINA (4). In addition, highly purified β1H by itself both directly inhibits the activity of C3b (4) and accelerates the rate of decay of the alternative pathway convertases, $\overline{C3bB}$ and $\overline{C3bBP}$ (2, 7).

Of great interest is the mechanism by which β1H exerts these effects. No proteolytic activity that can be directly ascribed to β1H has been found. Direct binding of β1H to C3b and subsequent steric interference with the interaction of C3b with factor B and/or C5 is the most straightforward explanation; two lines of evidence, fluid phase depletion and agglutination by antibody to β1H of EAC43 previously exposed to β1H (8), had indicated that such binding occurs. More recently, both this laboratory (9) and another (10) have presented further information about the binding of β1H to C3b-coated particles. The studies reported here give quantitative measurement of strength and valence of this binding, examine the influence of fluid phase C3 and C3b on it, and determine the effects that factor B (B) and properdin (P), which also bind to C3b, have on the binding of β1H to C3b-coated cells.

Materials and Methods

Reagents. Bio-Rad Ag-1-X-10 (chloride form), Bio-Rex 70, electrophoresis grade polyacrylamide, bis-acrylamide, and sodium dodecyl sulfate (SDS) were obtained from Bio-Rad Laborato-

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* Supported by grant AI 13049 from the National Institutes of Health and an Arthritis Clinical Research Center Grant from the Arthritis Foundation, New York. This is publication no. 120 from the Charles W. Thomas Fund, Medical College of Virginia.

‡ Post doctoral trainees supported by training grant T32 AM 07079 from the National Institutes of Health.

[1] *Abbreviations used in this paper:* A, rabbit antibody; B, factor B; β1H, β1H globulin; C3bINA, C3b inactivator; DGVB^{++}, equal volumes of GVB^{++} and D5W^{++}; D5W^{++}, 5% dextrose in water; E, sheep erythrocytes; FITC, fluorescein isothiocyanate; GVB^{++}, 0.1% gelatin veronal buffer; P, properdin; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; VBS, veronal-buffered saline.

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ries, Richmond, Calif. Radioiodide, both ^{131}I and carrier-free ^{125}I, was obtained from Amersham Corp., Arlington Heights, Ill. Trypsin and soybean trypsin inhibitor (SBTI) were obtained from Worthington Biochemical Corp., Freehold, N. J. Bovine serum albumin (Cohn Fraction V) was obtained from Calbiochem, San Diego, Calif. Las-R human complement C3 reagent kit was purchased from Hyland Diagnostics Div., Travenol Laboratories, Costa Mesa, Calif., and Sepharose 4B from Pharmacia Fine Chemicals, Inc., Piscataway, N. J.

Buffers. Isotonic veronal-buffered saline (VBS) containing 0.00015 M Ca^{++}, 0.0005 M Mg^{++}, and 0.1% gelatin (GVB^{++}) and 5% dextrose in water containing the same concentrations of divalent cations ($D5W^{++}$) were mixed in equal volumes; the resulting buffer, ionic strength of 0.065 and pH 7.4, is referred to as $DGVB^{++}$ and was used in all binding studies. GVB^{--} was made as described above except that the divalent cations were not present. A stock solution of 0.086 M EDTA, pH 7.5, was diluted in GVB^{--} to prepare 0.04 M EDTA GVB^{--}.

Component Purification. Guinea pig C1 (11) and human C2 (12) were prepared as published elsewhere. Partially purified factor B was obtained by a modification of the procedure of Götze and Müller-Eberhard (13). Normal human serum was adjusted to 40% Na_2SO_4 and the resulting precipitate was redissolved and subjected to chromatography on Bio-Rex 70 (13). Properdin was prepared by a modification in the procedure of Pensky et al. (14). The high salt eluate from zymosan was dialyzed against low ionic strength buffer, and the resulting P-containing precipitate was redissolved in VBS and subjected to chromatography on a Sephadex G-200 column. B (15) and P (16) were measured hemolytically as described elsewhere. Highly purified human C3 was prepared as described by Tack and Prahl (17), and β1H was prepared as described by Whaley and Ruddy (2). C3b was prepared from the purified C3 with trypsin and SBTI as described by Bokisch et al. (18).

Antisera. Antisera to C3, B, P, and β1H were induced in goats and were subsequently used in radial immunodiffusion (19) to determine the concentration of the various components. Pooled human serum, which had been previously calibrated against purified C3, B, P, and β1H, served as the standard. In radiolabeled preparations, concentrations of C3 and β1H were determined nephelometrically with a Hyland Laser Nephelometer PDQ Instrument (Hyland Diagnostics Div.) and a Hyland LAS-R human complement C3 kit for C3 determinations. When β1H was measured nephelometrically, doubling dilutions of pooled human serum (1:12.5 to 1:400) were used as standards. The 1:12.5 dilution was first filtered through a 0.4-μm Nucleopore filter (Nucleopore Corp., Pleasanton, Calif.) before further diluting. Rabbit anti-β1H was diluted with saline and subsequently an equal volume of phosphate-buffered saline (0.01 M PO_4^{--}, 0.15 M NaCl, pH 7.4) containing 4% polyethylene glycol was added to give a final antibody dilution of 1:60. 50 μl of standards or appropriately diluted unknowns were added to 1 ml antibody dilutions and after 1 h at room temperature were examined for light scatter in the nephelometer; 50 μl of the same samples added to 1 ml of saline served as blank controls.

The purity of the ^{125}I-labeled β1H (^{125}I-β1H) (see below) was also estimated by testing the ability of the preparation to be insolubilized with the monospecific goat anti-β1H. ^{125}I-β1H was mixed with an excess of goat anti-β1H, and after 30 min at 37°C the β1H-anti-β1H complexes were precipitated by adding a predetermined optimal amount of rabbit anti-goat IgG (Atlantic Antibodies, Westbrook, Maine). After 1 h at 37°C and overnight at 4°C, the complexes were washed three times with saline and the radioactivity remaining with the precipitate was measured. All β1H preparations tested in this manner were 80–87% precipitable by the anti-β1H.

Immunofluorescent Staining. The globulin fraction of goat anti-β1H was conjugated with fluorescein isothiocyanate (FITC) according to the method of Herbert et al. (20). The fluorescein to protein ratio (molar) of the final preparation was 2:1. Approximately 100 μg of β1H was covalently linked to Sepharose 4B (see below), and subsequently the specificity of the anti-β1H was confirmed in blocking experiments whereby fluorescent staining of the Sepharose-bound β1H was inhibited by reacting the FITC-anti-β1H with highly purified β1H.

The cells were examined for fluorescence by using a Zeiss photomicroscope II (Carl Zeiss, Inc., New York) with a HBO 200 light source, FITC excitation primary filter, and a 530-nm secondary filter.

Radioiodination. Highly purified β1H and C3 were radioiodinated with ^{125}I (carrier free) or ^{131}I by the use of the chloramine T procedure (21). Unbound iodide was removed by ion exchange chromatography with Bio-Rad Ag 1-X-10 (chloride form) and overnight dialysis versus VBS. In the final preparations the radioiodide was 90–98% precipitable with 10% trichloroacetic acid. The specific activities obtained were in the range of 2–5 × 10^6 cpm/μg and 0.1–1.0 × 10^6 cpm/μg for βlH and C3, respectively. Bovine serum albumin (Cohn Fraction V) was added to the stock solutions of the radiolabeled proteins, and storage was at −70°C. The correspondences between ^{125}I and ^{131}I counts and numbers of molecules of βlH or C3 were calculated from the specific activities of the iodinated proteins, using Avogadro's number and mol wt of 185,000 and 150,000 daltons (2) for C3 and βlH, respectively.

Cellular Intermediates. Sheep erythrocytes (E) were sensitized with rabbit antibody (A), and EAC4 were prepared and stored at −70°C in a glycerol-containing medium as described elsewhere (22). EAC4 were thawed as needed. EAC14 and EAC14^{oxy}2 were prepared by using guinea pig Cl and human C2 which had been oxidized with I_2 (23). EAC43 were prepared with nonoxidized C2 as described previously (15); the EAC43 did not lyse when exposed to a C3-9 source (rat serum diluted 1:15 in 0.04 M EDTA GVB^{--}). EAC14^{oxy}23 and EAC14^{oxy}2 ^{131}I-C3 were prepared by incubating EAC14^{oxy}2 in DGVB^{++} with purified C3 (either unlabeled or ^{131}I-labeled) for 30 min at 37°C followed by three washes with DGVB^{++}.

Counting Technique. Measurements of ^{125}I, ^{131}I, and ^{22}Na were made in a dual channel gamma counter (model 1185, Searle Analytic, Chicago, Ill.). When all three isotopes were used simultaneously, the samples were counted twice, first for ^{125}I and ^{22}Na and then for ^{131}I and ^{22}Na. Channel settings were adjusted such that 0.1% or less spillover of the lower energy isotope (order of energy: $^{22}\text{Na} > {}^{131}\text{I} > {}^{125}\text{I}$) into the higher one(s) occurred. The ^{125}I cpm was thus corrected for spillover of ^{22}Na and, when necessary, ^{131}I; ^{131}I cpm was corrected for ^{22}Na spillover. Background corrections were also made for all channels.

Radioactive Binding Assays. To avoid extensive manipulation of cells, ^{22}Na was used as a volume marker for unbound ^{125}I-βlH remaining with the cells. In a typical experiment, prepared cellular intermediates were incubated with ^{125}I-βlH for 15 min at 30°C in a vol of 1 ml. The cells were then sedimented by centrifugation and 0.1 ml of supernate removed. Approximately 90% of the remaining supernate was then removed by aspiration and the cells quantitatively transferred with DGVB^{++} into a clean tube. Radioactive determinations were then made on the supernatant aliquot and the cell pellet. Based on the assumption that the ratio of free ^{125}I-βlH and ^{22}Na in the incubation solution was constant, the amount of βlH bound to the cells was determined by the following formula:

$$\text{cpm } ^{125}\text{I} = \beta 1\text{H bound} = A - (x/y)(z)$$

where A is the total ^{125}I cpm in the cell pellet, x is the ^{22}Na cpm in the cell pellet, y is the ^{22}Na cpm in the supernate, and z is the ^{125}I cpm in the supernate. This method of calculation is similar to that described by Tsay and Schlamowitz (24). In situations where the ^{131}I-C3 was used to quantitate the amount of cell bound C3b, supernatant corrections with ^{22}Na were unnecessary since there was essentially no ^{131}I-C3 in the fluid phase.

In binding assays in which the objective was determination of binding parameters, EAC14^{oxy}23 or EAC14^{oxy}2 ^{131}I-C3 were incubated for 15 min at 30°C with various amounts (0.1-2 μg) of ^{125}I-βlH in a total vol of 1 ml, and subsequently the bound ^{125}I-βlH was determined as described above. The experimental data were then plotted according to the method of Scatchard (25).

$$r/c = nK - rK$$

where r represents the number of βlH molecules bound per cell (or alternatively per ^{131}I-C3b molecule), K is the average association constant, n the total number of binding sites per cell (or per C3b), and c is the concentration of free bindable ^{125}I-βlH. c was calculated as follows:

$$c = (\beta 1\text{H}_T)(\text{MB}) - \beta 1\text{H}_b,$$

where $\beta 1\text{H}_T$ is total βlH added (molecules/milliliter), MB is the maximal binding ability of the βlH preparation, and $\beta 1\text{H}_b$ is the number of molecules of βlH bound to the cell. In the final Scatchard plots, the best straight line for the experimental points was computed by the method of least squares using a Wang WCS-20 computer. K was obtained from the slope of this line and was converted to the more familiar liters per mole units by using Avogadro's number and a mol wt of 150,000 daltons (2) for βlH. The maximal number of βlH binding sites (n) was obtained from the intercept of the line with the abscissa. This intercept represents an infinite free concentration of βlH.

Other Analytical Procedures. Immunoelectrophoresis was performed by standard technique (26). SDS-gel electrophoresis with 7.5% separation gels was performed by the method of Laemmli

(27). Immunoadsorbents and β1H-Sepharose were prepared by linking the respective protein to Sepharose 4B by the cyanogen bromide procedure (28).

Results

Congruent Immunofluorescent Staining and Agglutination by Anti-β1H. Direct visual demonstration of both binding of β1H to EAC14^{oxy}23 and agglutination of such cells by anti-β1H was obtained. For this experiment, EAC14^{oxy}23 were prepared as described in Materials and Methods. One-half of the cell preparation (1.0 × 10^8 cells/ml) was then incubated for 15 min at 30°C with 12 μg β1H in a vol of 0.6 ml, and the other half with DGVB^{++} alone. After washing three times with DGVB^{++}, the two cell populations were remixed and subsequently allowed to interact for 30 min at 25°C with fluoresceinated anti-β1H. After washing in DGVB^{++}, the cell mixture was mounted on glass slides and examined by both phase and fluorescent microscopy. There was gross aggregation of some, but not all, of the cells. When examined by fluorescence microscopy, only the cells that stained for β1H by immunofluorescence were agglutinated, thus indicating that the agglutination was associated with the presence of bound β1H on the cells.

Relation between β1H Binding and Amount of Surface-Bound C3b. Using ^{125}I-β1H, the dependence of β1H binding on the presence of C3b was directly examined. Populations of cells bearing varying amounts of C3b on their surfaces were made by treating EAC14^{oxy}2 (5 × 10^7 per ml) with C3 concentrations ranging from 0 to 45.6 μg/ml for 30 min at 37°C. The cells were washed three times with DGVB^{++}, exposed to 0.9 μg/ml of ^{125}I-β1H for 15 min at 30°C, and then washed three more times with DGVB^{++}. After transferring to clean tubes, the amount of ^{125}I-β1H bound was determined. EAC14^{oxy}23 generated with increasing amounts of C3 bound increasing amounts of ^{125}I-β1H (Fig. 1). Approximately 29,000 molecules of ^{125}I-β1H were bound per cell at the highest input of C3; this represented 37% of the available ^{125}I-β1H.

Maximum Binding Ability of ^{125}I-β1H Preparations. The proportion of ^{125}I-β1H in a given preparation which was capable of binding to C3b was examined in the following experiment using an excess of cells relative to the concentration of ^{125}I-β1H. Varying numbers of EA, EAC14, EAC14^{oxy}2, or EAC14^{oxy}23 were incubated with 0.1 μg of ^{125}I-β1H for 15 min at 30°C, and the amount of binding determined by the ^{22}Na procedure described in Materials and Methods. This procedure was used to allow the detection of low affinity binding of the ^{125}I-β1H by preventing any loss of bound ^{125}I-β1H due to washing. Significant binding of the ^{125}I-β1H occurred only with EAC14^{oxy}23 intermediate (Fig. 2). Even with maximal numbers of these cells, however, only 48% of the ^{125}I-β1H was bound. In similar experiments with three other β1H preparations, maximal binding abilities ranged from 30 to 62%. Addition of fresh EAC14^{oxy}23 to supernates containing ^{125}I-β1H which had been previously exposed to EAC14^{oxy}23 did not result in any additional binding of the ^{125}I-β1H, indicating that the unbound material did not have the capacity to bind to cell-bound C3b. As indicated in Materials and Methods, 80–87% of the labeled β1H preparation was precipitated by monospecific goat anti-β1H. Kinetic experiments (data not shown) indicated that binding equilibrium was reached as early as 5 min at 30°C; thus, it is clear that some of the ^{125}I-β1H was not bindable. Possible reasons for this are

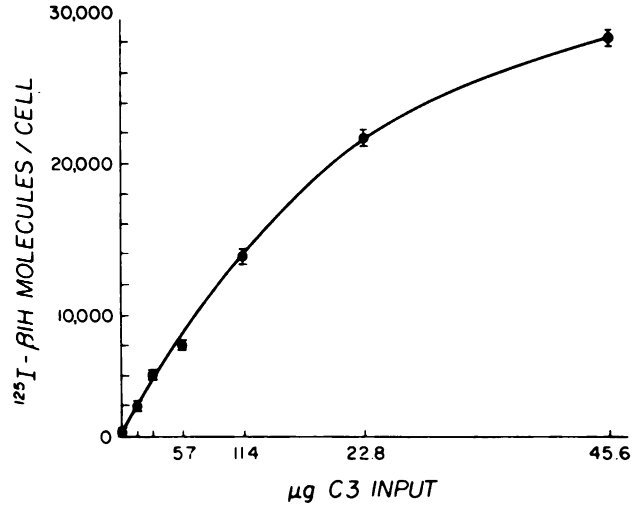

FIG. 1. ^{125}I-β1H binding by EAC14^{oxy}23 generated with increasing concentrations of C3. The bars indicate the standard error of the mean for triplicate determinations.

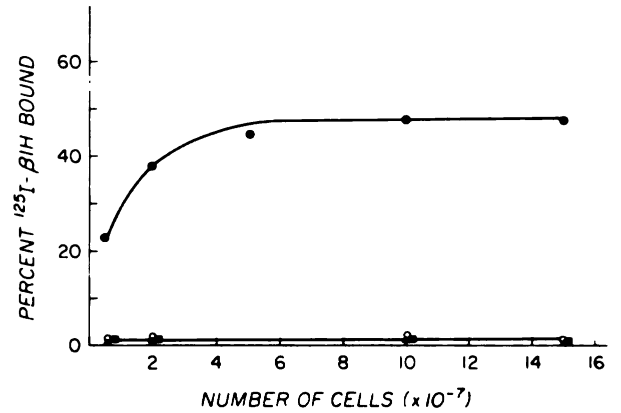

FIG. 2. Percent of ^{125}I-β1H bound by varying numbers of EA (▲), EAC14 (○), EAC14^{oxy}2 (■) or EAC14^{oxy}23 (●).

considered in the Discussion, but it should be noted here that in all of the quantitative analyses of ^{125}I-β1H binding, the maximum binding ability for the particular preparation was used in the calculations.

Quantitative Analysis of β1H Binding. When a constant number of EAC14^{oxy}23 (5×10^6 cells) is incubated for 15 min at 30°C with increasing concentrations of ^{125}I-β1H, and β1H binding initially increases rapidly and then tends to level off as the binding sites become saturated. A plot of the raw data from an experiment of this type is shown in Fig. 3a. Shown also is the same data corrected for the small amount of binding when EAC14^{oxy}2 was used instead of the EAC14^{oxy}23.

To estimate the binding constants of this reaction, the experimental data were subjected to the Scatchard analysis as described in Materials and Methods (Fig. 3b). Least squares analysis of the data gave an average association

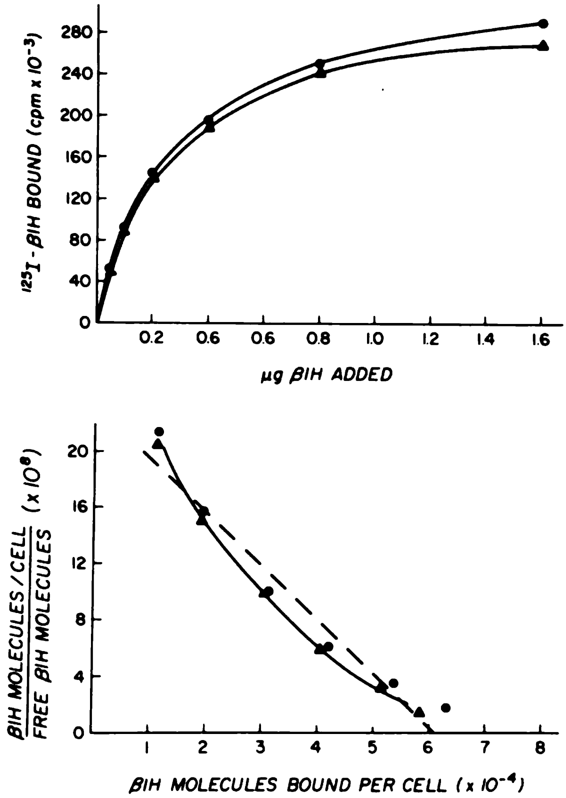

FIG. 3. Quantitative analysis of the interaction of varying concentrations of ^{125}I-β1H with EAC14^{oxy}23. In the upper panel the cpm ^{125}I-β1H bound is shown as a function of the amount of ^{125}I-β1H added; the lower panel is a Scatchard analysis of the same data (see text). In both, total binding to EAC14^{oxy}23 (●) and binding corrected for EAC14^{oxy}2 (▲) are shown. In the lower panel the dashed straight line has been fit by the method of least squares to the corrected data, and the unbroken line is a smooth curve drawn by hand through the same points.

constant (K) of 2.3 × 10^{9} L/M for this experiment; the range obtained in five similar experiments was 2–5 × 10^{9} L/M. Although the correlation coefficient for a straight line, obtained via the least squares analysis, was greater than 0.9 in all five experiments, the smoothest curve through the data points was always concave towards the abscissa (see Fig. 3b) rather than straight. As is also evident from Fig. 3b, the nonlinearity is not due to β1H binding to EAC14^{oxy}2, since correction for this binding does not greatly improve the straight line fit. Possible reasons for this deviation from linearity are given in the Discussion.

Extrapolation of the experimental data points in Fig. 3b to the abscissa indicates a maximum of 60–70,000 ^{125}I-β1H molecules bound per cell. In the Scatchard analysis shown in Fig. 4, EAC14^{oxy}2 ^{131}I-C3 were used to determine the number of β1H molecules bound per C3b molecule. Except for the ^{131}I-C3, the experimental conditions were identical to those used for Fig. 3. In Fig. 4, therefore, r represents the number of β1H molecules bound per C3b molecule. A K value of 3.9 × 10^{9} L/M was obtained from the slope of the line, and extrapolation to the abscissa indicates a value for r of 0.5, equivalent to an average of one molecule of β1H per two bound C3b molecules. The range for r in the three experiments done in this manner was 0.5 to 0.8 β1H per C3b; in no experiment was a 1 to 1 relationship achieved.

Influence of Fluid Phase C3 and C3b on the Binding of β1H to Cell Bound C3b. Evidence that interaction between C3b and β1H occurs in the fluid phase is provided by the data in Table I. For this experiment, tubes containing a constant amount (0.1 μg) of ^{125}I-β1H and increasing concentrations of either native C3 or C3b (prepared as described in Materials and Methods) or unlabeled β1H were incubated for 15 min at 30°C with 5 × 10^6 EAC43 in a total vol of 1 ml. The amount of ^{125}I-β1H bound was determined by the ^{22}Na procedure. Table I compares the concentration of unlabeled β1H required for 50% inhibition of ^{125}I-β1H binding with the amounts of C3 and C3b required for similar inhibition. At relatively high concentrations (approximately 1,000-fold molar excess over β1H), both native C3 and C3b inhibit binding of ^{125}I-β1H to C3b-bearing cells. Contamination of the C3 or C3b preparations with small amounts of β1H does not explain the results shown in Table I, since absorption with anti-β1H conjugated to Sepharose 4B has no effect on their inhibitory capacity. Similarly,

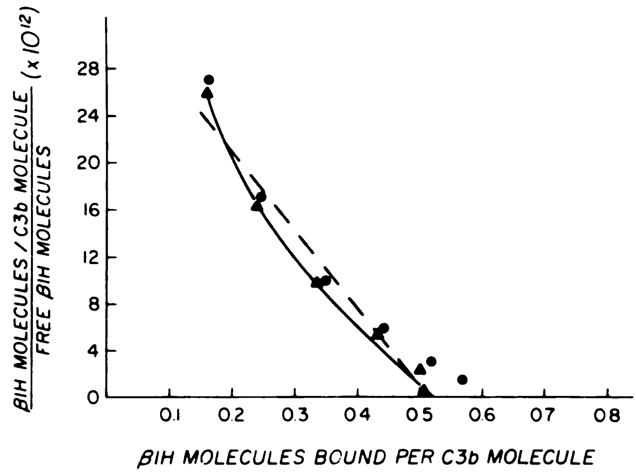

FIG. 4. Scatchard analysis of binding of ^{125}I-β1H to EAC14^{oxy}23 prepared with ^{131}I-C3. The latter allowed the enumeration of the numbers of C3b molecules which was 64,000/cell for this experiment. As in Fig. 4, total binding (●) and that corrected for EAC14$^{oxy}\underset{\sim}{2}$ (▲) binding are given. The dashed and smooth lines are also the same as in Fig. 3.

TABLE I

Inhibition of ^{125}I-β1H Binding to EAC43 by β1H, C3b, or C3

Inhibitor	50% inhibitory concentration	Relative molar inhibitory concentration (50%)*
	ng/ml	
β1H	125	1
C3b	141,250	942
C3	178,000	1,142

* Refers to the molar excess over β1H required for 50% inhibition.

contamination of the native C3 with C3b does not explain their approximately equivalent inhibitory capacity. No alteration in the electrophoretic mobility of the native C3 was seen by immunoelectrophoresis, and a single sharp band corresponding to the C3 α-chain was found when the preparation was examined by SDS-gel electrophoresis under reducing conditions.

Influence of Factor B on the Binding of β1H to Cell Bound C3b. As was the case for C3 and C3b, fluid phase B also inhibited the equilibrium binding of β1H to C3b-coated cells (Fig. 5). For this experiment, 0.1 μg of ^{125}I-β1H was mixed with increasing concentrations of either B, or for comparison, unlabeled β1H. EAC14^{oxy}23 (5×10^6 cells) were then added and after 15 min at 30°C, the amount of ^{125}I-β1H bound was determined by the ^{22}Na procedure. As seen in Fig. 5, B caused a dose-dependent inhibition of ^{125}I-β1H binding to the cells; on a molar basis 280-fold more B than unlabeled β1H was required for 50% inhibition of ^{125}I-β1H binding.

β1H has been previously shown to enhance the decay of factor B from EAC$\overline{43B}$ cells (2, 7). The experimental data shown in Fig. 6 demonstrated that the converse is also true in that B can cause enhanced release of β1H from EAC43 · β1H[2] cells. For this experiment increasing concentrations of B were incubated for 15 min at 30°C with 5×10^7 EAC43 · β1H bearing 4,100 molecules/cell of ^{125}I-β1H. Subsequently, the cells were washed three times with DGVB^{++} and the amount of ^{125}I-β1H remaining bound to the cells was determined. In the absence of B, 12% of the bound β1H was released; over and above this value, the percentage of bound ^{125}I-β1H released was directly proportional to the concentration of B added.

Influence of Properdin on β1H Binding to Cell Bound C3b. In view of the stabilizing effect that properdin has on the interaction between B and C3b (16), it was of interest to examine its influence on β1H binding. EAC43 cells (5×10^6) were incubated for 15 min at 30°C with various amounts of P and 0.1 μg of ^{125}I-β1H. As can be seen from the results shown in Table II, P caused a dose-dependent enhancement of binding of ^{125}I-β1H to EAC43 cells. When an amount of B sufficient to inhibit approximately 50% of the ^{125}I-β1H binding was added, the enhancing effect of P was lost.

In a separate experiment, a Scatchard analysis of ^{125}I-β1H binding to EAC43 cells in the presence and absence of a constant amount of P was performed. Two parallel curves were found, indicating that P did not change the affinity of β1H binding, but rather made more C3b sites accessible to the β1H.

Discussion

The purpose of the present work was to delineate clearly the C3b binding activity of β1H globulin and to investigate the influence that other proteins which bind to C3b have on the interaction of β1H with cell-bound C3b. Previous work had demonstrated agglutinability of EAC43 cells which had been incubated with β1H (8). The fluoresceinated anti-β1H used herein clearly demonstrates that this agglutination was due to bound β1H. Evidence that the β1H is binding to the C3b and not to the cell surface or other complement components is seen in the direct relation between the binding of ^{125}I-β1H, and the amount of

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[2] We propose this symbol, EAC43 · β1H to indicate an intermediate bearing bound β1H.

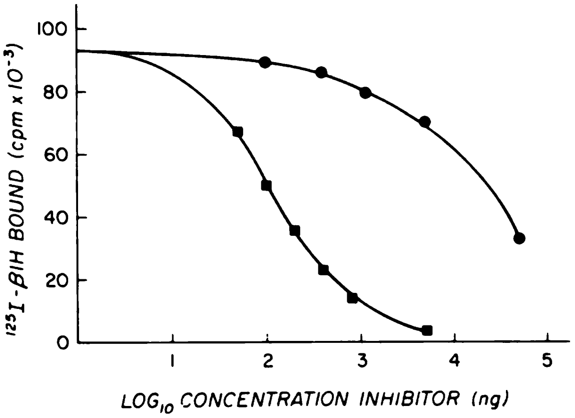

FIG. 5. Inhibition of ^{125}I-βlH binding to EAC14^{oxy}23 by unlabeled βlH (■) or B (●).

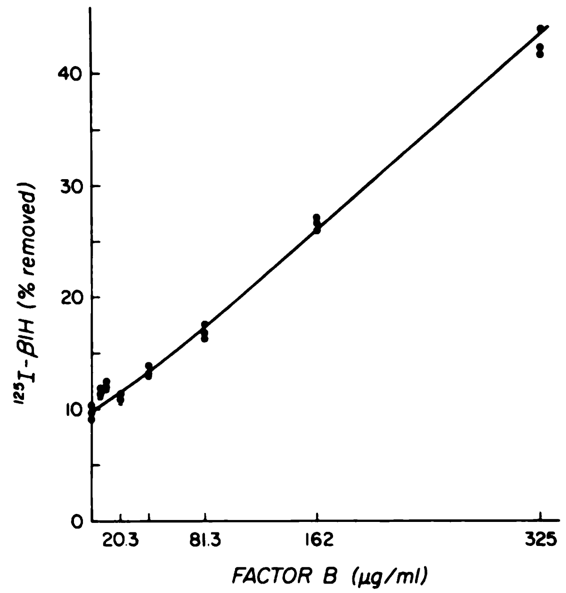

FIG. 6. Removal of ^{125}I-βlH from EAC43-βlH by varying concentrations of B. Values for triplicate determinations are shown.

C3 used to generate the EAC14^{oxy}23 (Fig. 1). In addition very little ^{125}I-βlH binds to the cellular intermediates EA, EAC14, or EAC14^{oxy}2 (Fig. 2).

In the majority of the binding experiments which used ^{125}I-βlH, ^{22}Na was used to correct for fluid phase (unbound) βlH. This procedure eliminates the necessity of washing and in addition allows the observation of weak binding interactions (24). However, the binding affinity of βlH turned out to be quite high; thus little difference was seen in experiments where three washes were used in place of the ^{22}Na procedure (data not shown).

The observation that not all of the ^{125}I-βlH was capable of binding to cell bound C3b, even when the C3b was in obvious excess (Fig. 2), was surprising. The most likely reason is simply that the C3b binding site on the βlH is somewhat labile, and while antigenically it can be recognized as βlH, the C3b

TABLE II

Enhancement of ^{125}I-β1H Binding to EAC43 by Properdin and Its Reversal by Factor B

Agent (μg added)	Molecules ^{125}I-β1H bound per cell	Percent of control
Buffer	7,643	100
P (0.01)	8,053	105.4
P (0.05)	8,537	111.7
P (0.25)	9,568	125.2
P (0.25) + B (40)	3,715	48.6
B (40)	4,069	53.2

binding ability is lost. It does not seem likely that the loss of binding ability is related to the radioiodination procedure; this level of labeling (2-8 × 10^6 cpm ^{125}I/μg β1H) represents an average less than 0.5 atoms of ^{125}I per β1H molecule which is a relatively low level of labeling. Also, competition experiments (Fig. 5 and Table I) indicated that unlabeled β1H was as effective as labeled preparations in binding to C3b. One other possibility to explain the decreased maximum binding would be release of C3b from the cells, and inhibition of β1H binding by the fluid phase C3b such as seen in Table I. However, this would be a viable possibility only if this released C3b was much more effective in inhibition than the trypsin-produced C3b used in Table I.

As discussed by DeMeyts et al. (29), determination of the maximal binding ability of the binding ligand in question is important with respect to further analysis of the binding data. If this is not done, the experimentally determined binding constants will be low (29). For this reason, in the calculation of the free β1H concentrations the total β1H added was adjusted to correspond to this experimentally determined maximal binding value.

The method of Scatchard (25) was chosen to analyze the β1H binding data. This approach has been used in many protein-ligand binding studies and, more recently, in cell receptor-protein binding situations (30, 31). The slope of the line is equal to −K and the intercept at the abscissa is maximum number of binding sites. As stated in Results, the best line through the data points (see Figs. 3a and 4) is concave towards the origin. There are three generally accepted reasons for deviation from linearity in this situation (29): (*a*) The binding site itself is structurally heterogeneous, as are the combining sites of various antibodies directed against the same antigen; the different sites might have different affinities. (*b*) Two or more entirely different classes of binding sites are present, each with different affinity for the ligand. This is frequently observed in experiments in which the amount of nonspecific binding is large. (*c*) Cooperativity between sites may result in changing affinity as sites become occupied. A concave plot is consistent with negative cooperativity (29), in which unfilled sites have a lower affinity for the ligand as increasing numbers of sites become filled.

For the interaction between β1H and C3b, site heterogeneity appears unlikely, since both proteins are supposed to be homogeneous. However, polymorphic forms of C3 are known to exist (32), and β1H has been shown to exhibit some microheterogeneity when subjected to isoelectric focusing (33); thus it is not possible to exclude site heterogeneity as the reason for the nonlinearity of the Scatchard plots in the present study. The second reason, independent classes of binding sites, has been effectively ruled out, since the second class of sites would be those present on EAC14^{oxy}2, and subtraction of the small amount of binding to this intermediate still does not linearize the plots. The final reason, negative cooperativity, appears the most likely: C3b is known to be deposited on the cell surface in a nonrandom manner (34); as the β1H binding sites on the C3b become occupied, the remaining C3b molecules, due to steric reasons, may be less accessible to additional β1H.

The maximum number of binding sites, n, may be underestimated when a concave Scatchard plot such as observed for the interaction of C3b and β1H is extrapolated in a linear fashion. Thus it is possible that the ratio of β1H to C3b might approach 1:1 if sufficiently high β1H concentrations were examined.

The functional consequences of the interaction between C3b and β1H in the fluid phase have been demonstrated by Fearon and Austen (35). Only a small amount of turnover of C3 and factor B was observed in mixtures of C3, B, $\bar{D}$, C3bINA, and β1H at concentrations similar to those found in serum. However, if β1H was left out of the reaction mixture both C3 and B were rapidly converted to hemolytically inactive components. Pangburn et al. (4), suggested that β1H was absolutely essential for C3bINA activity on C3b in fluid phase reactions. The inhibitory activity of native C3 and C3b on β1H binding seen in Table I is further evidence of the fluid phase interaction of β1H with C3 and C3b. In spite of the above, direct demonstration of complex formation between C3b and β1H in the fluid phase has not yet been achieved.

When examined for their effect of β1H binding to cell bound C3b, the two other C3b binding proteins, B and P, have essentially opposite effects. B both displaces bound β1H (Fig. 6) from the cell and inhibits the equilibrium binding of β1H to C3b (Fig. 5). This suggests that factor B and β1H interact with the same or closely adjacent sites on the C3b molecule. Others have attributed the ability of substances to activate the alternative pathway to their furnishing a protective "microenvironment," in which C3b bound to their surfaces is less accessible to inhibition by β1H (10, 35, 36). Since the interaction of B with C3b on these same surfaces is supposedly undiminished, these data suggest that B and β1H interact with different sites on C3b. There is no obvious explanation for this apparent paradox.

In the absence of B, P appears to increase the availability of C3b sites for β1H binding. It is evident from the work of Fearon and Austen (16) that P increases the hemolytic activity of factor B. This increase has been attributed to stabilization of the alternative pathway convertase (16), but properdin may cause increased equilibrium binding of B as well. The ability of properdin to extend the half-life of bound β1H is currently being investigated.

Summary

Purified β1H globulin (β1H) was shown to bind to C3b coated cells by both immunofluorescent and radioactive tracer techniques. With EAC43, the amount of β1H bound was directly proportional to the amount of C3 used to prepare the cells; EA, EAC14 and EAC14^{oxy}2 bound very small amounts of β1H. The C3b binding site on β1H was labile in that not all of the purified ^{125}I-β1H was

capable of binding to C3b, even when an excess of cell-bound C3b was present. Scatchard analysis of binding of β1H to C3b-coated cells indicated an equilibrium constant of 10^9 L/M. Deviations from linearity were regularly found on Scatchard analyses. This was consistent with the hypothesis that the β1H binding sites exhibit negative cooperativity in that as more sites become occupied, it becomes more difficult to fill the remaining sites. The stoichiometry of the reaction between C3b and β1H was examined using $EAC14^{oxy}23$ prepared with ^{131}I-C3 and β1H labeled with ^{125}I. Between 0.5–0.8 β1H molecules were bound per C3b molecule.

Other alternative pathway components influenced the binding of ^{125}I-β1H to cell bound C3b. Both C3b and native C3 inhibited binding of labeled β1H at an efficiency approximately 1/1,000 that of unlabeled β1H. Factor B inhibited binding with 1/280 the efficiency of unlabeled β1H. Properdin caused a dose-dependent increase in the binding of β1H; this enhancement was abrogated if B was also present in the reaction mixture. Scatchard analysis indicated that the enhancement of β1H binding by P resulted in an increased number of available binding sites rather than an increase in the affinity of binding.

The authors would like to express their appreciation to Mr. Don Purkall for excellent technical assistance and to Mr. Peter Evans for assistance with the nephelometry.

Received for publication 26 January 1978.

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SYNTHESIS AND SECRETION OF PLASMA PROTEINS BY EMBRYONIC CHICK HEPATOCYTES

Changing Patterns during the First Three Days of Culture*

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The production of plasma proteins is a major specialized function of the liver, plasma proteins representing roughly one-fifth of the hepatocytes' protein synthetic activity (1, 2). Study of this function not only will add to our understanding of protein biosynthesis in eukaryotic cells, but also will aid in elucidating the process of biochemical differentiation.

In this report the development of a model system for studying the regulation and differentiation of plasma protein synthesis is presented. A model system must be easily set up, reproducible and representative of the in vivo capacity of the hepatocyte to produce plasma proteins, and should allow experiments of several days duration as well as simple and fast measurement of plasma protein synthesis.

Plasma protein synthesis has been investigated in whole animals (1–6), perfused liver (7, 8), liver slices (9), liver cell suspensions (10, 11), and continuous cultures of liver (12) and hepatoma cells (13). For our purpose, whole animal studies are too ambiguous and the perfused liver system is too restricting in the number of conditions that can be varied within any one experiment. Work with whole animals, perfused livers, or liver slices poses difficulties in standardization. Suspensions of liver cells share with perfused livers the problem of being too short-lived. Preferring not to work with the relatively dedifferentiated continuous cell lines, we have concentrated on primary culture of chick embryo liver cells which had been developed in this laboratory and which maintain differentiated functions for several days (14–16).

Quantitation of output in studies of protein synthesis most commonly involves the use of radioactive isotopes and rather elaborate immunological procedures for identification. In contrast, our system eliminates the need for routine radioactive labeling by using a simple immunoassay to determine the synthesis of plasma proteins. The system combines the culture of chick embryo liver cells with Laurell's rocket immunoelectrophoresis technique (17) for identification and quantitation of the plasma proteins secreted into the medium. By an adaptation of this immunological procedure (18, 19), proteins can be assayed in small volumes of medium; sequential sampling and assay establish the kinetics of protein synthesis.

To permit meaningful conclusions to be drawn concerning protein synthesis, a number

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* Supported in part by grant GM-04922 from the National Institutes of Health, U. S. Public Health Service.

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of properties had to be established: (*a*) the type and number of cells and their viability in culture; (*b*) identification of the cultured cells that are responsible for plasma protein synthesis; (*c*) stability of the secreted proteins; (*d*) determination as to whether secretion reflects a release of stored proteins, or whether synthesis and secretion are continuous; (*e*) the overall time required for intracellular transport of plasma proteins synthesized in culture.

In this paper these points are addressed, and we further demonstrate that the rate of accumulation of a plasma protein in the medium is equal to the rate of its synthesis. We show that chick embryo liver cells synthesize proteins at in vivo rates for at least 72 h in culture and that the proportion of plasma protein synthesis to total hepatic protein synthesis is comparable to that observed in vivo. Synthesis of a number of plasma proteins is compared, and it is shown that decreasing synthesis with time in culture is specific to albumin alone. The advantages of this system in approaching several problems of biological interest have been discussed in an earlier report (18).

Materials and Methods

Culture Media and Reagents. The standard medium is modified Ham F-12 (16) containing 10% heat-inactivated (56°C for 30 min) and Millipore-filtered (Millipore Corp., Bedford, Mass.) fetal bovine serum (Grand Island Biological Co., Grand Island, N. Y.) and mycostatin (E. R. Squibb & Sons, Princeton, N. J.), 25 U/ml. To improve fibrinogen detection, 15 μg heparin sodium/ml (Connaught Laboratories, Toronto, Canada) was added to the standard medium where so indicated. Serum-free medium is modified Ham F-12 supplemented with insulin (crystalline, bovine pancreas, Sigma Chemical Co., St. Louis, Mo., 24.3 IU/mg) at a concentration of 0.2 μg/ml.

Acetoxycycloheximide was obtained from the Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Md. Actinomycin D was obtained from Merck Chemical Div., Merck & Co., Inc., Rahway, N. J.

L-[4,5-^{3}H]leucine, sp act 6 Ci/mmol, was obtained from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y. A ^{3}H-labeled L-amino acid mixture, as well as Omnifluor and Protosol, were from New England Nuclear, Boston, Mass.

Primary Culture of Liver Parenchymal Cells. The culturing procedure described previously (14) was used with minor modifications. Briefly, perfused livers dissected from 16-day-old chick embryos were trimmed of connective tissue and disaggregated by gentle mechanical and enzymatic treatment (15). 5 ml of concentrated cell suspension, derived from three livers and containing single cells and aggregates of 2–10 cells, was routinely diluted 20-fold in medium before inoculation of 2, 5, or 12 ml, respectively, into 35-, 55-, or 80-mm diameter Falcon Petri dishes (Falcon Plastics, Div. BioQuest Oxnard, Calif.). In this way, approximately the same ratio of surface area to volume of inoculum was attained, resulting in similar cell densities after plating. Less than an hour elapsed between excision of liver and initial incubation of the cells in a 5% CO_2-air atmosphere at 38°C. All viable cells attached within 2 h to the plastic surface of the dish. Culture medium with cell debris, nonviable cells, and erythrocytes could be aspirated and replaced with fresh medium as early as 2 h after plating. The ratio of adhering cells to supporting medium was usually in the range of 1,000,000/ml. Variations made in this general procedure are noted in legends to the figures.

Antisera. Polyspecific antisera to adult and 17-day embryonic chicken serum, as well as monospecific antisera to chicken albumin and fibrinogen, were produced in rabbits according to standard methods (20). The plasma proteins designated "C" and "M"[1] were followed with a trispecific antiserum whose preparation, characterization, and usage is described elsewhere.[2] The antiserum contains anti-albumin, anti-C, and anti-M antibodies. Other antisera used in this study were prepared against various chicken antigens and were kindly provided by the following colleagues: antiplasminogen, Dr. Allan R. Goldberg (The Rockefeller University); anti-12-day embryonic plasma proteins, Dr. Edwin M. Weller (University of Alabama; 21); antilipovitellin, Dr. Robert F. Goldberger (National Institutes of Health; 22); antiovalbumin, Mr. George Kuzmycz (The Rockefeller University); anti-alpha-macroglobulin, Dr. Deane F. Mosher (University of Wisconsin); and the antisera against the apoproteins of very low density lipoprotein and against apo-A, the major apoprotein of high density lipoprotein, Dr. David J. Kudzma (University of Miami).[3]

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[1] The functions of "C" and "M" have not been identified; for simplicity, C and M are used in this paper.

[2] G. Grieninger and J. Pindyck. The application of a modification of rocket immunoelectrophoresis to the study of plasma protein synthesis in cultured hepatocytes. Manuscript in preparation.

Anti-total chicken plasma proteins, hereafter referred to as anti-TCPP,[4] is a mixture of antisera against various chicken antigens: anti-adult chicken serum, anti-17-day embryonic chicken serum, antifibrinogen, antialbumin, antilipovitellin, anti-12-day embryonic plasma proteins, and the antiserum against the apoproteins of very low density lipoprotein. Anti-mouse albumin was kindly provided by Dr. Hans P. Bernhard (Biocenter, University of Basel).

The titer and specificity of antisera used were determined by electroimmunoassay and crossed immunoelectrophoresis (18). No cross-reactions were observed between antisera to chicken plasma proteins and proteins present in 10% fetal bovine serum.

Electroimmunoassay and Crossed Immunoelectrophoresis. The electroimmunoassay (rocket immunoelectrophoresis) of secreted plasma proteins was performed as described previously (18); a more detailed treatment of the application of the method will be published elsewhere.[2] In the assay procedure, 3 μl of culture medium, containing secreted proteins, was used without preliminary concentration.

Crossed immunoelectrophoresis was performed as described on concentrated samples of medium in which monolayers had been maintained (18), except that the immunoplates were stained with Coomassie Blue (23) for better photographic contrast.

Determination of Intracellular Plasma Protein Levels by Electroimmunoassay. The monolayers of cells were washed with Earle's balanced salt solution minus magnesium and calcium and detached by adding Na_2EDTA (0.02%) in the same solution. A cell pellet was obtained by centrifugation at 180 *g* for 5 min. Disruption of the cells was accomplished by first freezing then homogenizing the thawed sample at 4°C in 1 ml of sodium barbital buffer (44 mM, pH 8.6) followed by addition of Triton X-100 to a final concentration of 0.5%. Samples were withdrawn for cellular protein determination. After cell debris was removed by centrifugation, the concentrations of cytoplasmic plasma proteins were determined on 3 μl of supernate by electroimmunoassay. The detergent did not disturb the immunoassay or the determination of cellular protein. As an alternate to this procedure, the cells could be disrupted by sonication for 5 min and were found to yield identical intracellular plasma protein levels.

Radioactive labeling of hepatic proteins. In several experiments, short labeling periods were desired, and labeling was terminated by washing the cells four times with Earle's balanced salt solution and then adding fresh, label-free culture medium. Samples were withdrawn at the times indicated and mixed with an equal volume of 16% (wt/vol) trichloroacetic acid and the resulting precipitates were filtered on Whatman GF/C glass fiber filters (Whatman, Inc., Clifton, N. J.) and washed with 8% trichloroacetic acid containing an excess of the unlabeled amino acid(s). To the dried precipitates a toluene-based scintillation fluid was added containing 0.4% (vol/vol) Omnifluor, 9% (wt/vol) Protosol, and 0.3% (vol/vol) ammonium hydroxide; the samples were counted in a liquid scintillation counter.

In long-term labeling experiments, cells were exposed to a mixture of ^{3}H-labeled amino acids for a period of 24 h. The culture medium was centrifuged to remove any debris and anti-TCPP serum was used to precipitate the radioactively labeled plasma proteins without addition of carrier protein. Preliminary studies had shown that 0.2 ml of the antisera mixture quantitatively precipitated 20 μg of chicken plasma proteins from a final volume of 0.4 ml. Hence, 0.2 ml of anti-TCPP was routinely incubated with 0.2 ml of culture medium for 0.5 h at 25°C and overnight at 4°C. Labeled albumin in the medium was specifically immunoprecipitated in an analogous fashion with anti-chicken serum albumin. Immunoprecipitates were filterod in the cold on two overlaid

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[3] D. J. Kudzma, J. B. Swaney, and E. N. Ellis. Apolipoproteins of the chicken: effect of estrogen administration. Manuscript submitted for publication.

[4] *Abbreviations used in this paper:* TCPP, total chicken plasma proteins.

Whatman GF/C glass fiber filters and washed thoroughly with cold phosphate-buffered saline containing an excess of the unlabeled amino acid(s) used during labeling. It was found that two filters were required to quantitatively retain the immunoprecipitate. The filters were then dried and counted as described above.

The measurement of label incorporated into cellular protein was accomplished by scraping the cells from the dishes with the aid of a rubber policeman into 8% (wt/vol) trichloroacetic acid and processing the precipitates as described above.

Estimation of Protein, DNA, and Cell Number. Cells were washed and detached from the culture dish with EDTA as described above. The cell suspension was centrifuged, and the cell pellet was resuspended in a small volume of the EDTA solution. Portions of this cell suspension were employed in determination of protein by the method of Lowry et al. (24), and DNA by the method of Burton (25). The cell number was determined in a hemocytometer.

Determinations of cellular protein, DNA content, the incorporation of radiolabeled precursors, and the amount of plasma proteins detected with immunochemical techniques were made on at least three culture dishes.

Results

Hepatocyte Culture. Cells, prepared and inoculated as described in Materials and Methods, formed a monolayer (14) at a density, determined in several experiments, between 2 and 3 × 10^5 cells per cm^2. The cell count was performed on a suspension of cells removed from the dish and was confirmed by counting nuclei in photographs of monolayers. Floating and loosely adhering cells are considered nonviable because they fail to produce detectable plasma proteins when removed and reinoculated 2 h after the original inoculation. Based on our observation that the protein content of 3 × 10^6 cells amounts to approximately 1 mg, we estimate that 30% of the hepatocytes in the intact liver are recovered in culture as viable, attached liver cells.

At the high cell densities used, mitotic figures were scarcely ever seen. Cell count, cellular DNA, and cell protein were found to be constant for at least 72 h in culture demonstrating that the cells do not divide and do not increase in mass. These findings are consistent with studies that showed that mitotic activity decreases in the intact liver both with the age of the chick embryo and with higher cell densities (26). We suggest that in these cultures the embryonic hepatocytes are arrested in the G_1 phase of the cell cycle as are mature hepatocytes in vivo (27). Support for this claim is derived from a calculation of the amount of DNA per nucleus based on determinations of cellular DNA and the number of cells. When corrected for binucleated cells (26), a value of 2.5 pg of DNA per nucleus was obtained which agrees with the diploid amount reported for hepatic cells of the domestic fowl (28).

The presence of nonparenchymal cells, particularly fibroblasts, in culture was negligible at the high cell densities used, a phenomenon also observed by others (29, 30). With immunofluorescent staining, it could be shown that uniformly all hepatocytes contained the various plasma proteins studied so far.[5] Fibroblasts were not found to contain plasma proteins with this method. Furthermore, in medium from cultures of fibroblasts on days 1, 2, and 3, no secreted plasma proteins could be detected by electroimmunoassay, even when the medium was concentrated 10-fold. This shows that any fibroblasts contaminating the hepatocyte culture do not contribute to the output of plasma proteins.

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[5] R. G. Kalb and G. Grieninger. Immunofluorescent localization of plasma proteins in cultured embryonic chick hepatocytes. Manuscript in preparation.

Stability of the Secreted Plasma Proteins in the Culture Medium. Chick embryo liver cells secrete plasma proteins which accumulate in the culture medium (18). When medium, in which cells had been maintained for 24 h and which contains the secreted plasma proteins, was incubated for another 24 h under the usual culture conditions but now in the absence of cells, no decrease was observed by electroimmunoassay in the amount of three major plasma proteins: albumin and the two plasma proteins designated C and M[1] (18; see Fig. 1 for electrophoretic mobilities). No decreased radioactivity in total plasma proteins (labeled in culture as described in Table I*a*) was found by quantitative precipitation with the antibody mixture, anti-TCPP, after a similar 24-h incubation of medium in the absence of cells. When protein synthesis was inhibited by acetoxycycloheximide (31), a potent derivative of cycloheximide, and the cells and medium were left to incubate at 38°C, the levels of albumin, C (Fig. 2), M, and fibrinogen (data not shown) remained unchanged for 20 h. Furthermore, by maintaining the hepatocytes in chemically defined, serum-free medium, it could be demonstrated that the cells do not secrete detectable levels of active proteases (A. R. Goldberg and G. Grieninger, unpublished observations.). These experiments strongly suggest that the cells do not break down the secreted plasma proteins to any significant extent under the culture conditions described. Because the plasma proteins are stable after they have been secreted, the rate of accumulation of a plasma protein in the medium represents its rate of secretion by the cells.

Secreted Proteins Represent Plasma Proteins. The nature of proteins secreted by the embryonic chick liver cells in culture was studied in samples of culture medium collected after a 24-h exposure of the cells to a [^{3}H]amino acid mixture. A measure of secreted proteins was obtained by trichloroacetic acid precipitation of aliquots of the medium. In other aliquots of the same medium, radioactivity in plasma proteins was assessed by precipitation with the antisera mixture anti-TCPP which in preliminary experiments recognized virtually all (adult and embryonic) antigens present in chicken plasma obtained from various developmental stages. The amount of acid-precipitable radioactivity in the medium was found to be identical with the amount immunoprecipitable with anti-TCPP. In other aliquots of the medium, the absence in the immunoprecipitate of proteins other than chicken plasma proteins was demonstrated by precipitating mouse albumin with anti-mouse albumin with the reagents present at concentrations corresponding to those of the reagents in the anti-TCPP precipitation. From the complete correspondence found between acid-precipitated and immunoprecipitated material, it can be concluded that the hepatocytes in culture secrete plasma proteins almost exclusively. Furthermore, it is possible in this cell culture system to interpret trichloroacetic acid-insoluble radioactivity in the medium as representing labeled plasma proteins secreted by the cells.

Spectrum of Secreted Plasma Proteins. Two-dimensional or crossed immunoelectropherograms revealed that a wide spectrum of plasma proteins accumulated in the medium during the first, second, and third days of culture (Fig. 1). The size and intensity of the individual immunoprecipitates ("rockets") indicate the amount of specific protein present. We have shown that the intensity of the immunoprecipitate reflects the amount of antigen by using

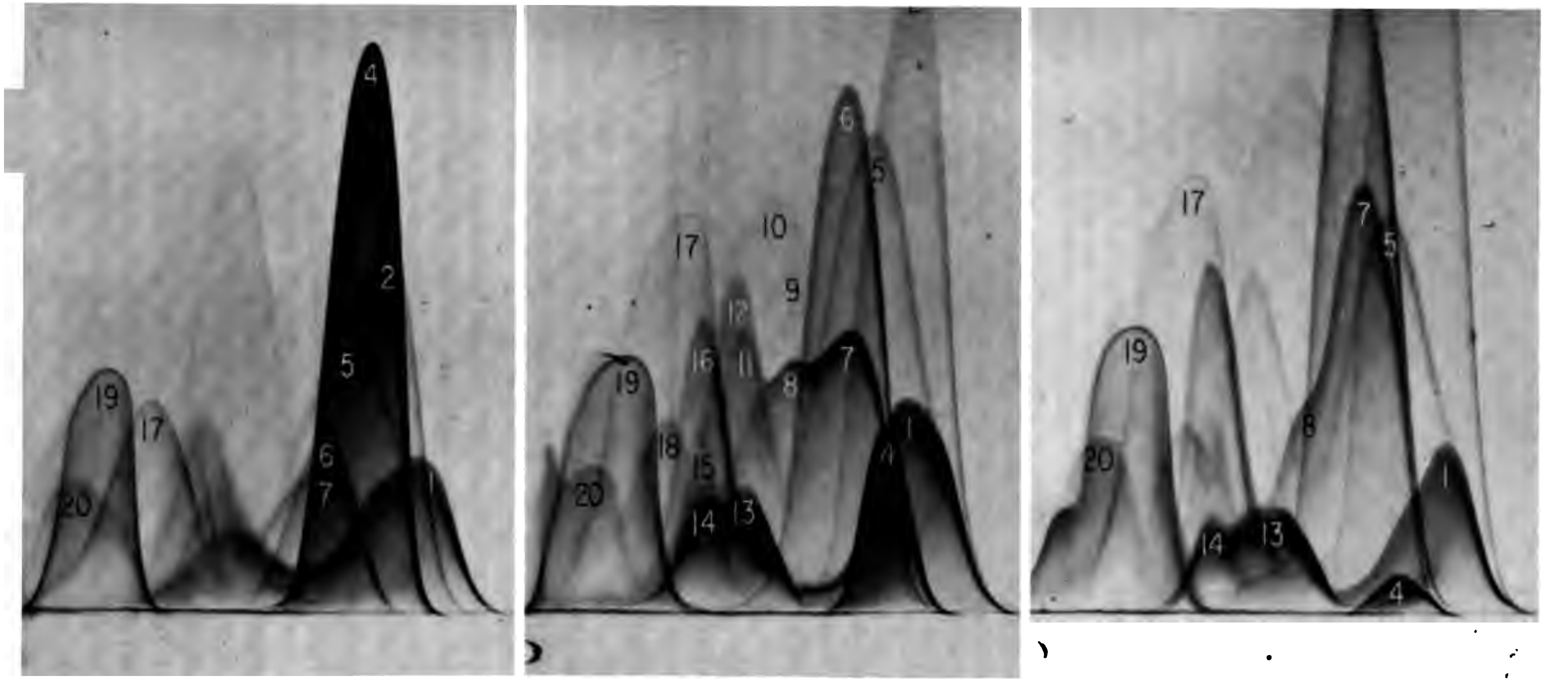

FIG. 1. Crossed immunoelectrophoresis of plasma proteins secreted on days 1, 2, and 3 of culture. Cells were plated in standard medium in 80-mm diameter dishes. The medium was changed 2.5 h later and every 24 h thereafter. At 2.5, 26.5, and 50.5 h, selected sets of dishes were transferred to serum-free medium and incubated for an additional 24 h. The medium was then collected, centrifuged at 500 *g* for 10 min, and concentrated 85-fold. Concentrated medium (5 μl), containing plasma proteins corresponding to the levels secreted by 4.2 × 10^6 cells in 24 h, was applied in the sample well visible in the lower left corner of each panel. Electrophoresis in the first dimension was performed from left to right and in the second dimension from bottom to top. The antibody-containing gel contained 10% (vol/vol) anti-adult chicken serum, i.e. 14 μl/cm^2. The following plasma proteins have been identified immunologically: peak 2 = plasma protein C, peak 4 = albumin, peak 6 = plasma protein M, peaks 13 and 14 = lipoproteins, peak 15 = alpha-macroglobulin, peak 19 = transferrin. (*a*) Secretion between 2.5 and 26.5 h: day 1. (*b*) Secretion between 26.5 and 50.5 h: day 2. (*c*) Secretion between 50.5 and 74.5 h: day 3.

TABLE I

Synthesis of Plasma Proteins during 3 Days of Culture

Radioactivity in plasma proteins determined by	Labeling period	Day 1	Day 2	Day 3
(*a*) Immunoprecipitation	24 h	100	88	79
(*b*) Acid precipitation	5 min	100	88	98

Cells were plated and maintained in 2 ml of medium in 35-mm diameter dishes. Total plasma protein synthesis was determined by two independent methods. Rosults obtained for days 2 and 3 are expressed relative to the values obtained for day 1. (*a*) Long-term labeling. At 4 h postinoculation, and every 24 h thereafter, the medium was replaced with fresh medium containing 15 μg heparin/ml. Different sets of dishes were exposed to a ^{3}H-amino acid mixture added to the medium at 2.5 μCi/ml at 4, 28, and 52 h (day 1, day 2, and day 3, respectively). After 24 h of incubation, total plasma protein in the culture medium – amounting to approximately 15 μg – was immunoprecipitated with anti-TCPP as described in Materials and Methods. The value obtained for the first day was 3,450 cpm/0.2 ml of medium. (*b*) Short-term pulse. 1 h before the addition of label, the cells were washed with medium and then placed in 1.0 ml of medium. At 24, 48, and 72 h of culture (day 1, day 2, and day 3, respectively), different sets of dishes were pulsed for 5 min with 15 μCi of [^{3}H]leucine/ml. After the labeling period, the cells were washed and reincubated in 1.0 ml of label-free medium for 80 min, whereupon trichloroacetic acid-precipitable radioactivity was determined in the entire volume of culture medium. The value obtained for the 1st day was 3,756 cpm/ml of medium.

radioactively labeled plasma proteins and comparing electropherograms with their respective autoradiograms (18). The antiserum used in Fig. 1 was prepared against adult chicken serum and was chosen for its balanced titers of antibodies against various plasma proteins, thereby permitting good discrimination between individual peaks. When compared to anti-TCPP, the antiserum was found to precipitate >80% of the plasma proteins secreted by the cells in culture. The anti-adult chicken serum does not recognize several secreted plasma proteins, namely, fibrinogen, plasminogen, a fetal protein (FP-1) and a phosphoprotein.

In the central panel (b) of Fig. 1, at least 20 different plasma protein peaks can be distinguished and are numbered consecutively from right to left. To match the corresponding peaks in panels a and c, the respective medium samples were directly compared by tandem crossed immunoelectrophoresis (32) with the medium used in b. With this technique, immunologically related peaks derived from two antigen samples fuse to form double peaks, thus permitting direct comparison of different crossed immunoelectrophoresis patterns. With the use of specific antisera, certain peaks have been identified: peak 2 = plasma protein C, peak 4 = albumin, peak 6 = plasma protein M, peaks 13 and 14 = lipoproteins, peak 15 = alpha-macroglobulin. Peak 19 was identified as transferrin by tandem-crossed immunoelectrophoresis with conalbumin, a protein that has a similar polypeptide constitution and is immunologically indistinguishable from transferrin (33).

The proteins secreted by the cultured hepatocytes compare well with plasma proteins present in embryonic blood. By tandem-crossed immunoelectrophoresis it could be demonstrated that most of the proteins present in plasma derived from 15- to 19-day-old chick embryos were secreted in culture with the notable

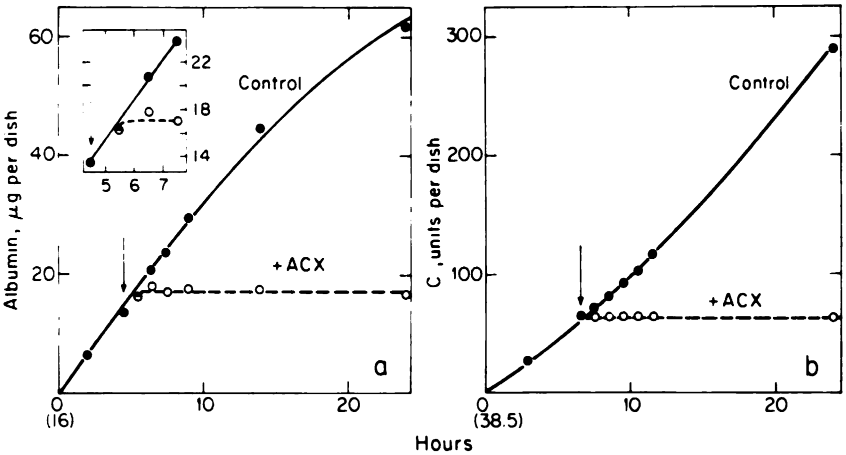

FIG. 2. Inhibition of secretion of the plasma proteins albumin and C by acetoxycylohex-imide (ACX). Cells, equivalent to 1.8 mg cellular protein, were kept in 55-mm diameter dishes. The secretion of albumin and C was determined by electroimmunoassay and is expressed as micrograms of albumin or units of C in the culture medium per culture dish. 10 U is equivalent to about 1 μg of protein. The experiments in a and b were performed on dishes established in the same culture. (a) Time-course of albumin secretion. Zero time represents the time fresh medium was added 16 h after plating. Acetoxycycloheximide is added 4.5 h later (arrow) to a concentration of 0.15 μg/ml. The *inset* enlarges both scales to show the time period immediately after the addition of acetoxycycloheximide. (b) Time-course of C secretion. Zero time represents the time fresh medium was added 38.5 h after plating. Acetoxycycloheximide is added 6.5 h later (arrow) to a concentration of 0.15 μg/ml.

exception of immunoglobulins, ovalbumin, and another fetal protein, FP-2 (data not shown).

With crossed immunoelectrophoresis, we were able to observe dramatic changes in the spectrum of secreted plasma proteins, the most prominent occurring between the 1st and 2nd days. In Fig 1 a, the height and intensity of peak 4 indicates that albumin was the major protein secreted early in culture. Later in culture, plasma protein M and the lipoproteins (peak 6 and peaks 13 and 14, respectively, Fig. 1 c) predominated. Fig. 1 also shows that the albumin secreted in fresh medium during day 3 was only a trace of that secreted on day 1. This decrease in albumin secretion was paralleled by an increase in the amount of other plasma proteins secreted.

Electroimmunoassay with monospecific or oligospecific antisera provided a simpler and more precise means for quantification of particular plasma proteins. This is illustrated with assays to determine albumin and plasma protein C, which were performed on unconcentrated culture medium samples taken as described in the legend to Fig. 1. The amount of albumin secreted on days 1, 2, and 3 was found to be 10.5, 1.8, and 0.3 μg/4.2 × 10^5 cells. The same population of cells produced plasma protein C levels of 9.5, 22, and 40 U on these respective days. 10 U correspond to approximately 1 μg of protein.

De Novo Synthesis of Secreted Plasma Proteins in Culture. Secreted plasma proteins became radioactivity labeled when chick embryo liver cells were exposed to radiolabeled amino acids in culture, indicating *de novo* synthesis

(18). The contribution of *de novo* synthesis to the amount of a plasma protein secreted in culture was studied under conditions in which protein synthesis was inhibited. Acetoxycycloheximide was added to the medium at a concentration of 0.15 μg/ml, a concentration that inhibited the incorporation of labeled amino acids into total hepatocellular proteins by about 95%. As seen in Fig. 2, the accumulation of albumin and plasma protein C was interrupted by the addition of acetoxycycloheximide. These results suggest that *de novo* protein synthesis is required to maintain secretion.

To learn more about the relationship of intracellular proteins to their secreted counterparts, we compared the amounts of plasma proteins released in the medium with the levels maintained intracellularly (Fig. 3). It can be seen that changes in the secretion rate of each protein were reflected in changes in its cellular level: the intracellular amount of albumin decreased with the decline in its rate of secretion (Fig. 3a), whereas the intracellular amount of C increased with the rise in its rate of secretion (Fig. 3b). The initial substantial intracellular C level is not understood. Inasmuch as the actual amounts of both albumin and C inside the cells were small relative to the quantities exported (<5% of the daily secretion), one can conclude that the secreted proteins measured in the culture medium represent *de novo* synthesis rather than a release from storage pools developed before culture. These findings strongly suggest that the intracellular levels of plasma proteins reflect a steady state between synthesis and secretion.

Stimulation of the rate of secretion of a plasma protein by some given factor(s) might be expected to be accompanied by elevated cellular levels of that protein (i.e. establishment of a new steady-state level). This was explored by evaluating the effect of actinomycin D (Fig. 3a), a drug found to stimulate albumin secretion (34). As shown, the stimulation of the rate of albumin secretion is, indeed, reflected in a simultaneous increase of the intracellular albumin level.

An independent estimate of the level of albumin in the cells can be obtained from experiments described above. Immediately after the addition of acetoxycycloheximide, the cells continue to secrete albumin for 1 h before accumulation ceases – 3.7 μg/dish (see inset, Fig. 2a), an amount equivalent to 2 μg/mg cell protein. Peters and Peters (2), working with the liver of the intact rat, similarly found that in the absence of protein synthesis, secretion of previously synthesized albumin continues. We assume that the albumin (2 μg/mg cell protein) that continues to be secreted by the embryonic chick liver cells in the presence of acetoxycycloheximide is the steady-state albumin present intracellularly at the time the inhibitor was added (20.5 h). This is confirmed by direct measurement of cellular albumin in the experiment described in Fig. 3a, i.e. after 20 h of culture the intracellular albumin amounts to approximately 2 μg/mg cellular protein.

The failure to observe a parallel gradual cessation of accumulation in the case of plasma protein C can be attributed to this circumstance: the intracellular level of C at 46 h (2 U/mg cell protein, Fig. 3b) corresponds to <4 U/dish, a value too low to be resolved in Fig. 2b. Hence C secretion appears to stop abruptly upon the addition of acetoxycycloheximide.

Intracellular Transit Time. The rapidity of secretion after synthesis is

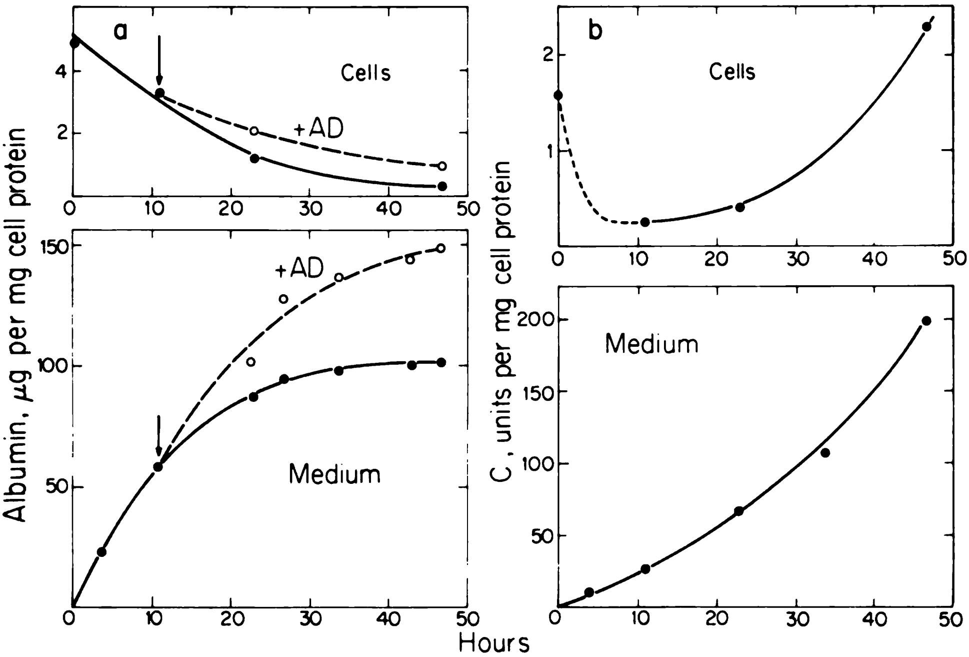

FIG. 3. Intracellular and secreted levels of albumin and plasma protein C. The cells were prepared as described in Materials and Methods except that the concentrated cell suspension was washed with medium by centrifuging at 180 *g* for 5 min before further dilution. The cells were maintained in 80-mm diameter dishes and culture medium was changed at 4, 11, and 23 h. Cellular protein was determined at 11, 23, and 47 h and was found to remain constant at 4.2 mg per dish. Albumin and plasma protein C levels in the cells as well as in the medium were determined by electroimmunoassay and are expressed per milligram of cellular protein. Zero time values were determined on a sample of the cell inoculum. The data shown in a and b for control cells (no drug) were obtained from the same dishes. (a) Albumin, in micrograms per milligram of cell protein, intracellularly (upper panel) and secreted into the medium (lower panel; cumulative levels). At 11 h, actinomycin D (AD) was added to some dishes to a final concentration of 0.25 µg/ml (arrow). The actinomycin D was replenished in these dishes at the next medium change which occurred 23 h after plating. Addition of the drug resulted in a reduction of cell protein from 4.2 mg to 3.8 and 3.4 mg per dish at 23 and 47 h, respectively. ●——●, Control cultures; O--O, cells treated with actinomycin D. (b) Plasma protein C, in units per milligram cell protein, intracellular (upper panel) and secreted into the medium (lower panel; cumulative levels). 10 U is equivalent to about 1 µg of protein.

limited by the time required for the transport of the proteins along the secretory pathway from their assembly at the polysomes to their release from the cell. The intracellular transit time of the total population of plasma proteins in cultured chick hepatocytes was explored in the experiment described in Fig. 4. The cells were labeled with [^{3}H]leucine for 70 min, a time found in preliminary experiments to be sufficient for establishing a steady state of radiolabel in intracellular plasma proteins. The cells were then washed and incubated again in unlabeled medium. At frequent time intervals, the trichloroacetic acid-precipitable radioactivity accumulating in the medium was determined. In the figure, it can be seen that within 60 min the accumulation of acid-precipitable

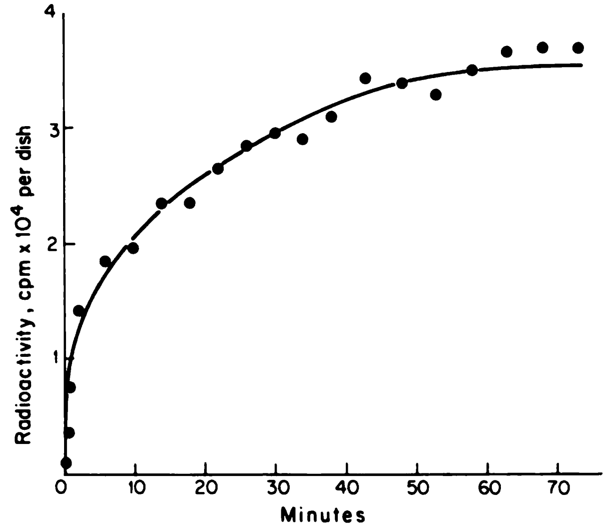

FIG. 4. Time-course of secretion of plasma proteins previously labeled to steady state. Cells were plated in 35-mm diameter dishes. After 3 h and again 20 h later, medium changes were performed. The cells were then labeled for 70 min with 25 μCi [^{3}H]leucine per dish. At the end of this period, they were washed as described in Materials and Methods and incubated, as before, for various lengths of time but in label-free medium. Zero time dishes were placed immediately on ice without further incubation. Trichloroacetic acid-precipitable radioactivity in the medium in cpm per dish (i.e. per 2 ml), as shown on the ordinate, was determined at each time indicated on the abscissa. Each point represents the average of measurements obtained with 0.8-ml samples from four dishes. The same half-time of accumulation of acid-insoluble radioactivity was observed when excess unlabeled leucine was present during the wash and in the medium.

counts has ceased. We interpret these data to mean that all labeled plasma proteins have left the cells within 1 h after the termination of the labeling period and that about 80% of the overall plasma proteins have been secreted within 30 min of their synthesis.

The time required for completed transit of albumin can be estimated from an experiment described earlier. From Fig. 3a, it is possible to say that at any given time the albumin present in the cultured hepatocytes constitutes less than the amount secreted per hour. In other words, the intracellular albumin level constitutes <5% of the daily secretion of this protein. From this, one may estimate that albumin molecules, once synthesized, should all be exported within 1 h.

Rate of Total Plasma Protein Synthesis in Culture. Total plasma protein synthesis in culture was quantified in two ways. First, cells were exposed to a mixture of radiolabeled amino acids for 24 h beginning at different times in culture, and chicken plasma proteins in the medium were quantitatively immunoprecipitated (Table I*a*). The radioactivity detected in the immunoprecipitate is a measure of the daily synthesis of total plasma proteins. The use of long-term labeling is valid in this case because of the stability of the secreted plasma proteins. A slight decrease in incorporation is observed over 76 h.

Second, cells were labeled with [^{3}H]leucine for only 5 min at different times in culture, washed, and transferred to unlabeled medium. After 80 min, the trichloroacetic acid-precipitable radioactivity in this medium was determined. Because the half-time of intracellular transit for the total population of plasma proteins was found to be about 10 min (Fig. 4), a labeling period of 5 min was chosen to assure that all labeled plasma proteins were still contained within the cells at the medium change. The subsequent 80-min period was shown to be sufficient for complete secretion of all the plasma proteins labeled during the 5-min pulse (compare with Fig. 4). In Table I*b*, it can be seen that acid-precipitable radioactivity, representing labeled plasma proteins, does not change substantially over 3 days. The results of both experiments demonstrate that the nonproliferating chick hepatocytes produce overall plasma protein at a nearly constant rate for 3 days in culture.

Relationship of Plasma Protein Synthesis to Total Hepatic Protein Synthesis. An estimate of the percentage of total hepatic protein synthesis in culture contributed by plasma protein synthesis was obtained in experiments designed as follows. After a 5-min exposure to [^{3}H]leucine (pulse period), the washed cells from one group of dishes were used to determine radiolabel incorporated into cellular protein which served as a measure of total hepatocellular protein synthesis. Fresh label-free medium was added to the washed cells from a second group of dishes that were then incubated for 80 min to permit secretion of all proteins that had become labeled during the pulse. At the end of this time, the medium was assayed for trichloroacetic acid-insoluble radioactivity to provide a measure of total plasma protein synthesis. In several independent trials, plasma proteins represented about 20% of the hepatocellular proteins synthesized during the pulse. This relationship was maintained over a 3-day culture period.

During the 1st culture day, 90 μg of albumin was synthesized per milligram of cell protein as determined by electroimmunoassay (Fig. 3 and reference 18). By quantitative immunoprecipitation of labeled plasma proteins in the medium with anti-TCPP as well as with antialbumin, it was shown that albumin synthesis comprises almost 50% of plasma protein synthesis during that period (see also Fig. 1*a*). Hence, total plasma protein synthesis, previously shown to be constant (Table I), is approximately 200 μg/mg of cell protein daily. Because plasma protein synthesis, in turn, is about 20% of total hepatic protein synthesis, we conclude that the liver cells in culture synthesize protein in the range of 1 mg/day per mg of cellular protein.

Discussion

This paper demonstrates the suitability of embryonic chick liver cells in primary culture as a model system for the study of plasma protein synthesis.

Cells in Culture. Primary cultures of embryonic chick hepatocytes have the advantage of being both rapidly achieved and highly reproducible. The cells generated from only a few embryo livers are sufficient to permit multiple observations and, because of the excellent yield of cultured cells per liver, we believe that the cultures contain a population of hepatocytes that are representative of those in the intact tissue. Optimal plasma protein production by the cells occurs even at the high cell densities chosen for routine cultures.[6] The homogeneously populated monolayers of nonproliferating hepatocytes remain viable for at least a week. After the 4th day of culture, we observed a general decrease in protein synthesis, probably due to prolonged maintenance of the cells in a confluent monolayer. However, throughout the first 3 days, the amount of plasma proteins synthesized was found to be constant (Table I). Moreover, the amount of plasma proteins secreted in a given experimental period can be related to a fixed cell number and cell mass because the cells are not dividing. These features of the culture permit its straightforward application to study of the rate of plasma protein secretion over prolonged periods of time.

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[6] It was found that the secreted amounts of albumin, C and M, are directly proportional to the number of cells (per dish) over a range of cell densities from 0.1 to 3.3 × 10^5 cells per cm^2.

Secretory Proteins. Because all the labeled proteins that are secreted into the medium, as measured by trichloroacetic acid precipitation, are precipitable with the antisera mixture anti-TCPP, it can be concluded that the hepatocytes in culture secrete plasma proteins almost exclusively. The secreted proteins studied are stable in the medium. To date, 24 of these plasma proteins have been distinguished and quantified (Fig. 1). Among them are most of the plasma proteins represented in the blood of the chick embryo with the exception of certain proteins, namely, the immunoglobulins, ovalbumin, and one particular fetal protein (FP-2), which are presumably synthesized in the embryo by either the lymphocytes or the yolk sac. In addition, the cultured embryonic liver cells synthesize several adult-type plasma proteins (corresponding to peaks 6, 7, and 20 in Fig. 1) which are absent in the developing embryo (18) and which appear in the blood of the chick only after hatching (G. Grieninger, unpublished observations). Apparently these embryonic hepatocytes differentiate in culture.

Synthesis and Secretion. About 80% of the plasma proteins were found to be secreted within 30 min of their synthesis. In other words, the time required for plasma protein movement along the secretory pathway is short, the overall half-time being <10 min (Fig. 4). Our findings are in excellent agreement with studies performed on intact animals (35, 36).

In the cultured chick hepatocytes, cellular levels of plasma proteins are low. The cells secrete all plasma proteins within 1 h of synthesis (Fig. 4), from which it follows that internal steady-state levels are equivalent to <5% of the daily secretion. Intracellular levels determined directly for albumin and C support this conclusion (Fig. 3).

Inasmuch as intracellular levels vary directly with the changing rates of secretion (Fig. 3), it can be concluded that the degree of secretion of a plasma protein is determined by its degree of synthesis. In our routine experiments, therefore, the rate of secretion into the medium, as determined by sequential immunoassay of accumulating plasma protein, gives a valid estimate of the rate of synthesis of that plasma protein.

Hepatic Protein Synthesis. The rate of total protein synthesis (including plasma proteins) in embryonic chick liver cells in culture was estimated to be approximately 1 mg/mg cell protein per day. This is in excellent agreement with the values derived for total hepatic protein synthesis in the intact chicken (M. Gruber, personal communication), rat (1), and mouse (37). The daily rate at which the liver cell synthesizes the equivalent of its own protein content seems to represent an upper limit of the rate of protein synthesis in the liver. For example, injection of a chicken with estradiol causes the liver to produce large quantities of the egg yolk precursor protein, vitellogenin, comprising up to 10% of total protein synthesized (38); but total protein synthesis remains constant (38, 39). These observations suggest that with respect to protein synthesis the liver is working at full capacity and that the rate of protein synthesis is limited by the amount of protein synthetic machinery. This is further supported by the observation that most liver ribosomes are active (37).

In light of the evidence that the liver cells in culture do not grow, all of the protein synthesized must be exported or turned over. In experiments reported here, exported proteins were estimated to be 0.2 mg plasma proteins/mg of cell protein per day as well as 20% of total protein synthesis. It follows that in culture 80% of the cellular protein is replaced per day. Similar replacement rates have been calculated for the intact liver (40, 41) using data obtained from the rat and mouse.

Albumin synthesis. Albumin is the major plasma protein synthesized early in culture. During the 1st day, albumin comprises nearly 50% of total plasma protein synthesis, i.e. 10% of hepatic protein synthesis in culture. These values completely confirm in vivo data from other systems: albumin comprised about half of total plasma protein synthesis in the intact rat (1) and 10% of hepatic protein synthesis in the chicken (37).

The decline in albumin secretion in culture was accompanied by falling intracellular levels (Fig. 3). With immunofluorescent staining it could be shown that the albumin level decreases uniformly in all cells.[5] To explain the declining internal levels, protein degradation during intracellular transport, specific for albumin, was considered. However, two lysosomotropic drugs, chloroquine and neutral red, which inhibit intracellular degradation of protein (42), did not stimulate albumin secretion. In addition, no radiolabel in cellular albumin was detectable after a short pulse at 72 h (G. Grieninger, unpublished observations), at a time when albumin secretion has virtually ceased, hence we conclude that the decreasing rate of secretion reflects a true decrease in albumin synthesis.

When viewed against the simultaneous increased synthesis of other plasma proteins, particularly the induction of adult-type plasma proteins, the dwindling rate of albumin synthesis cannot be attributed to a general dedifferentiation of the liver cell's ability to synthesize plasma proteins. Furthermore, it is unlikely that the change observed in the spectrum of plasma proteins synthesized is due to the appearance of new cell populations selected by the culturing process because we have shown that the cells do not proliferate in culture.

The specific decrease of albumin synthesis is apparently introduced by the culturing process and is not related to embryonic development because it is also observed with liver cell cultures obtained from either 10- or 19-day-old chick embryos (G. Grieninger, unpublished observations). This "phenotypic change" (43) should, therefore, be reversible or preventable with appropriate modification of the culture conditions. Recent experiments have confirmed this hypothesis, demonstrating continued albumin synthesis when cell cultures are initiated and maintained in a chemically defined medium in the absence of fetal bovine serum (44). We are currently exploring the nature of this apparent suppression of albumin synthesis.

We observed that for 72 h the rate of total plasma protein synthesis remains constant in culture despite drastic changes occurring in the synthesis of individual plasma proteins during this time. Under the described culture conditions, the enhanced synthesis of several plasma proteins, such as C and M, occurs most strikingly during the first 2 days of culture, just when the most dramatic decrease of albumin synthesis is observed (Fig. 1), suggesting a possible relationship between these events: increased synthesis of other plasma proteins seems to compensate quantitatively for declining albumin synthesis. Based on the evidence presented and indications that each hepatocyte has the capacity for synthesizing the entire spectrum of plasma proteins,[5] we interpret our results in the following way: in culture, the hepatocyte changes its program for the synthesis of specific plasma proteins while continuing the overall production of plasma proteins at full capacity. The molecular basis for this change of program will be addressed in a subsequent paper.

Summary

A simple model system is described for studying synthesis of plasma proteins. The system is based on chick embryo hepatocytes in primary monolayer culture which synthesize a broad spectrum of plasma proteins and secrete them into the culture medium. The secreted proteins are stable and consist almost exclusively of plasma proteins. The cultured cells are nonproliferating hepatic parenchymal cells whose cell mass remains constant in culture. By a modification of Laurell's rocket immunoelectrophoresis, the secreted plasma proteins can be detected in nanogram amounts in 3 μl of unconcentrated culture medium. Kinetics of secretion are obtained by sequential assay of proteins accumulating in the medium.

In this system it is demonstrated that: (*a*) intracellular plasma protein levels are equivalent to <5% of the daily secretion; (*b*) synthesis and secretion are continuous; and (*c*) the overall half-time for plasma protein movement along the secretory pathway is <10 min. From these results, it follows that the rate at which the plasma proteins are secreted gives a valid estimate of their rate of synthesis. This feature of the culture and the sensitivity of the assay allow routine measurements of plasma protein synthesis without disruption of the cells and without the use of radioisotopes. It is shown, furthermore, that the overall rate of plasma protein synthesis in cultured hepatocytes is constant over a 3-day period and is similar to that of the intact liver. 3,000,000 cells, containing 1 mg cell protein, synthesize 0.2 mg of plasma proteins daily, amounting to one-fifth of hepatocellular protein synthesis. Under the conditions used, albumin synthesis steadily decreases with culture time whereas the synthesis of many other plasma proteins increases. The observed phenotypic changes and reorganization of plasma protein synthesis illustrate how the system may be exploited for studying the regulatory processes governing plasma protein synthesis.

We wish to thank John Viets, Jay Klarsfeld, and Tsan-Yang Liang for their expert technical assistance during these studies. We also wish to thank Dr. Johanna Pindyck for her many valuable discussions and Dr. Merrill W. Chase for his incisive criticism and advice. We are grateful to Kathe M. Hertzberg for her invaluable support and critical examination of the manuscript.

Received for publication 8 February 1978.

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ANTIGEN REQUIREMENTS FOR INDUCTION OF B-MEMORY CELLS

Studies with Dinitrophenyl Coupled to T-Dependent and T-Independent Carriers*

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A cooperative interaction between thymus-derived (T) helper cells (T_H)[1] and antibody-producing (B) cells is required for an optimal antibody response to most antigens, i.e. T-dependent (TD) antigens. (1). By contrast, another group of antigens, the so-called T-independent (TI) antigens, apparently does not require T_H to optimally activate B cells (1, 2). However antibody responses induced by TI antigens differ markedly from those induced by TD antigens. In particular, most TI antigens elicit antibody which is solely or primarily of the IgM class (1–5) and multiple injections of TI antigens do not elicit IgG memory responses (1, 2, 6–8).

The general inability of TI antigens to induce memory in IgG-producing B cells (B_γ) has several possible explanations. For example, the population of B cells which responds to TI antigens (9) may be inherently incapable of undergoing differentiation to memory B_γ cells. This may be due to the fact that TI antigens do not activate T_H which might be required for such differentiation to occur (10–12) or because TI antigens preferentially activate suppressor T cells (T_s) (4, 13) which might actively suppress B-cell differentiation. On the other hand, TI antigens may be able to induce B-cell differentiation but may be unable to activate T_H which are required for expression of the IgG memory response (14–16). The present study was undertaken to differentiate among these possibilities by determining whether B cells from mice primed with TI antigens could produce IgG memory responses when T_H (primed by a TD antigen) were provided at the time of secondary challenge.

Materials and Methods

Mice. CAF_1 mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. Female mice, 8–12 wk old, were used for all experiments.

Antigens. The thymus-dependent antigens used for this study included dinitrophenylated ovalbumin (DNP_{30}-OVA) and dinitrophenylated keyhole limpet hemocyanin (DNP-KLH) contain-

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* Supported in part by grant AI-12830 from the National Institutes of Health, U. S. Public Health Service.

[1] *Abbreviations used in this paper:* DNP-KLH, dinitrophenylated keyhole limpet hemocyanin; DNP-OVA, dinitrophenylated ovalbumin; LPS, lipopolysaccharide; PFC, plaque-forming cells; TD, T-dependent; T_H, thymus-derived helper cells; TI, T-independent; TNP, trinitrophenyl.

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ing 31 DNP groups per 10^5 molecular weight of KLH. KLH (Pacific Bio-Marine Supply Co., Venice, Calif.) was used to stimulate carrier reactive T cells. The trinitrophenyl (TNP) or DNP haptens were coupled to TI carriers to provide the TI antigens used here. TNP was coupled to lipopolysaccharide (LPS) (Difco Laboratories, Detroit, Mich.) as described by Jacobs and Morrison (17). DNP was coupled to type III pneumococcal polysaccharide (S3) as described by Mitchell et al. (18) and DNP-Ficoll was a gift of Dr. Joseph Davie, Washington University, St. Louis, Mo. The preparations of TNP-LPS, DNP-S3, and DNP-Ficoll used here were considered to be TI since they elicited similar responses in both athymic nude and thymus-bearing littermate control mice (H. Braley-Mullen, unpublished results). Moreover, these antigens have been shown to be TI in other studies (2, 7, 8, 13).

Priming. Mice to be used as B-cell donors were primed with the above DNP- or TNP-conjugated antigens 1-2 mo before use in adoptive transfer experiments. Antigens were injected intraperitoneally, with 10^9 *Bordetella pertussis* organisms. Unless indicated otherwise, the amounts of antigen used for priming were: 100 μg DNP-OVA, 100 μg DNP-KLH, 10 μg TNP-LPS, 0.6 μg DNP-S3, and 2 μg DNP-Ficoll. Preliminary experiments established that these doses elicited optimal DNP-specific primary responses in CAF_1 mice. Mice used as T-cell donors were primed 1-2 wk before use with 100 μg KLH plus 10^9 pertussis organisms.

Depletion of T Cells from Donor Spleen Cells. Spleen cells from mice primed with the above DNP (TNP) conjugates were depleted of T cells by treatment with anti-Thy 1 serum and complement as previously described (10, 19). The specificity of the anti-Thy 1 serum was established previously (10). For each experiment B cells were obtained from a pool of at least three spleens from nonimmunized (normal) or primed donors.

Preparation of T Cells. Spleen cells from KLH primed mice were enriched for T cells by passage over nylon wool columns (20) as previously described (19).

Adoptive Secondary Response. Donor T and B cells were injected i.v. into 650 rads irradiated (19) CAF_1 mice. All recipient mice received 5-10 × 10^6 T cells and 10 × 10^6 B cells. Preliminary control experiments established that mice repopulated with B cells alone produced very few DNP-specific PFC (<1,000 PFC/spleen for B cells from mice primed with DNP-KLH and <200 PFC/spleen for B cells from mice primed with any of the other antigens). Mice repopulated with KLH primed T cells alone always produced <1,000 PFC/spleen. Mice were challenged i.v. with 10 μg DNP-KLH immediately after cell transfer. DNP-specific plaque-forming cells (PFC) were enumerated 7 days later (shown by preliminary experiments to be the time of the optimal DNP-specific IgG response).

PFC Assay. The number of DNP-specific PFC in recipient spleens was determined by the slide modification of the Jerne plaque assay (21) by using TNP-coupled horse erythrocytes (TNP-HRBC) as indicator cells (22). The number of indirect (IgG) PFC was determined by subtracting the number of PFC developed with a rabbit antiserum to mouse IgG_1 and IgG_2 (19). The developing antiserum was used at a concentration (1/300) determined to develop optimal numbers of IgG PFC in sheep erythrocyte immunized mice. The antiserum did not inhibit direct PFC at this concentration. All data are expressed as PFC/spleen but the conclusions would not differ if data were expressed as PFC/10^6 cells.

Results

TI Antigens are Unable to Prime B_γ Memory Cells. The first experiment was designed to determine whether DNP-specific B_γ memory cells could be primed by DNP coupled to TI carriers if primed T_H were provided at the time of secondary challenge. CAF_1 mice were primed with the TD antigen DNP-KLH or with the TI antigens DNP-Ficoll, TNP-LPS, and DNP-S3. 1 mo later splenic B cells from these mice and from normal unprimed mice were transferred to irradiated recipients with T_H cells from KLH primed mice. All mice were challenged with DNP-KLH (Fig. 1). Clearly, only those mice which were primed with the TD antigen DNP-KLH had B cells which could produce significant DNP-specific IgG antibody. B cells from mice primed with the TI antigens produced no more IgG than B cells from normal unprimed mice. This was true whether or not the pertussis adjuvant was given at the time of priming

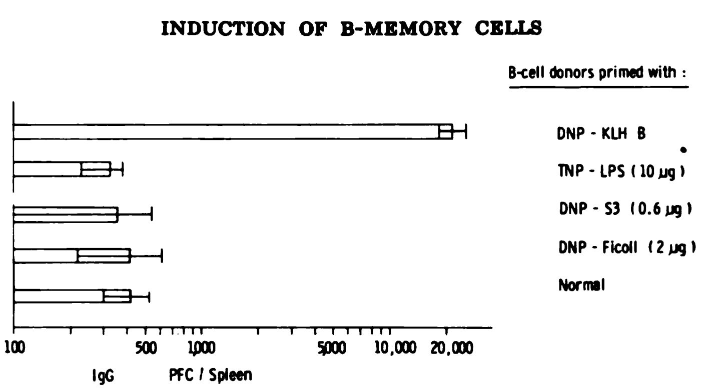

FIG. 1. Secondary IgG PFC responses of B cells from mice primed with TI or TD antigens. Irradiated recipients were repopulated with 10×10^6 T cells from mice primed with KLH and 10×10^6 B cells from mice primed with the antigens indicated in figure. B cells designated as normal were from nonimmunized mice in this and all subsequent figures. Recipient mice were challenged with 10 μg DNP-KLH. Values shown are mean DNP-specific indirect (IgG PFC/spleen ± SEM (5–10 mice/group).

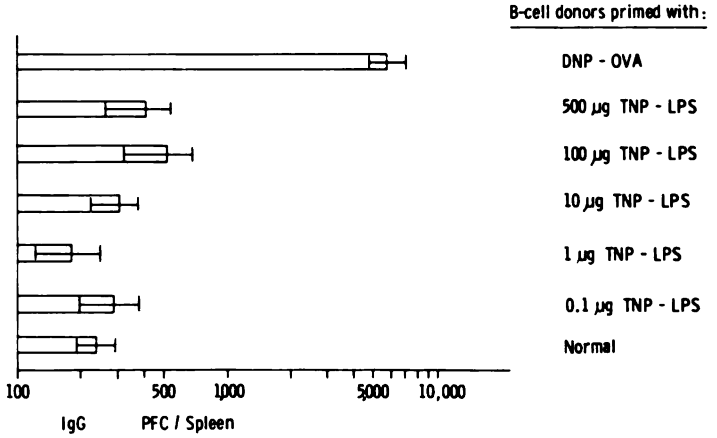

FIG. 2. Effect of dose of TNP-LPS on B-memory cell induction. Irradiated recipients were repopulated with 5×10^6 T cells from mice primed with KLH and 10×10^6 B cells from mice primed with the amounts of antigen indicated in the figure. Recipient mice were challenged with 10 μg DNP-KLH. Values shown are mean DNP-specific indirect (IgG) PFC/spleen ± SEM (6–12 mice group).

(not shown). Mice primed with DNP on a TD carrier (DNP-OVA) different from that used for challenge also produced good secondary IgG responses (see below).

Effect of Various Doses of TI Antigens. In the experiment described above mice were primed with doses of TI antigens which elicited optimal primary DNP-specific IgM responses. Since a different amount of antigen might be optimal for priming B_γ memory cells, groups of CAF_1 mice were primed with 0.1–500 μg TNP-LPS or with the TD antigen, DNP-OVA. 1 mo later splenic B cells from these mice and from normal unprimed mice were transferred to irradiated recipients with KLH primed T_H and DNP-KLH (Fig. 2). Only the mice which were primed with DNP-OVA had B cells which could produce significant IgG antibody. B cells from mice primed with either low or high doses of TNP-LPS produced only slightly more IgG than B cells from normal unprimed

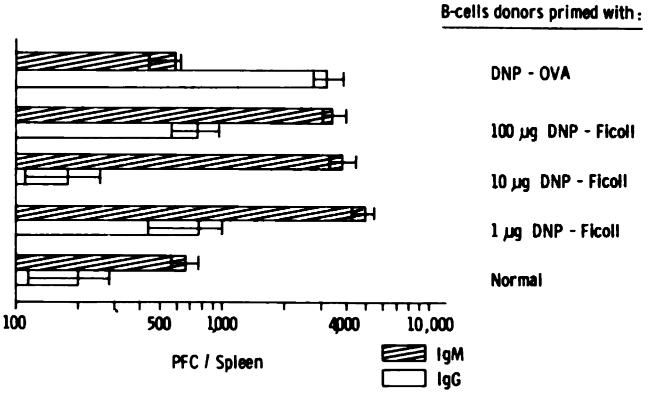

FIG. 3. Effect of dose of DNP-Ficoll on B-memory cell induction. Irradiated recipients were repopulated with 5×10^6 T cells from mice primed with KLH and 10×10^6 B cells from mice primed with the amounts of antigen indicated in the figure. Recipient mice were challenged with 10 µg DNP-KLH. Values shown are mean DNP-specific direct (IgM) and indirect (IgG) PFC/spleen ± SEM (five mice/group).

mice. Similar results were obtained when B-cell donors were primed with 1, 10, or 100 µg DNP-Ficoll, i.e. none of these doses of antigen could optimally prime B_γ memory cells (Fig. 3).

IgM Memory Responses. In the experiments described above we were concerned primarily with the effects of TI antigens on B_γ memory cells. The secondary IgM responses of B cells of mice primed with the TD antigens DNP-OVA and DNP-KLH or with the TI antigens TNP-LPS and DNP-S3 were no different from the IgM responses of B cells from normal mice i.e. these antigens apparently induced no IgM memory (data not shown). However, DNP-Ficoll, although incapable of inducing IgG memory, did induce a substantial IgM memory response in all experiments (Fig. 3).

TI Antigens Do Not Suppress the Induction of B_γ Memory Cells. Since TI antigens are unable to prime B_γ memory cells (Figs. 1–3) it is possible that they activate T_s which could suppress the differentiation of B cells to B_γ memory cells. To test this possibility, CAF_1 mice were injected simultaneously with a TI antigen (TNP-LPS) and a TD antigen (DNP-OVA). 1 mo later B cells from these mice, from normal mice, or from mice primed with only one of the antigens were transferred to irradiated recipients with KLH primed T_H and DNP-KLH (Fig. 4). Neither 10 or 100 µg of TNP-LPS suppressed the induction of B_γ memory cells by DNP-OVA. In other experiments 2 µg DNP-Ficoll also did not suppress the induction of B-memory cells by DNP-OVA (not shown).

Discussion

The results presented here show that B cells from mice primed with TI antigens do not produce significant IgG memory responses when memory T_H cells (induced by a TD antigen) are added at the time of secondary challenge. Thus the general inability of TI antigens to induce IgG memory responses (2, 6–8) is not due solely to the inability of these antigens to activate T_H which are required for the expression of memory (12, 14, 15). TI antigens also must be unable to activate a particular cell which is needed for the induction of IgG memory. This cell may be the B_γ precursor cell itself or the T_H cell.

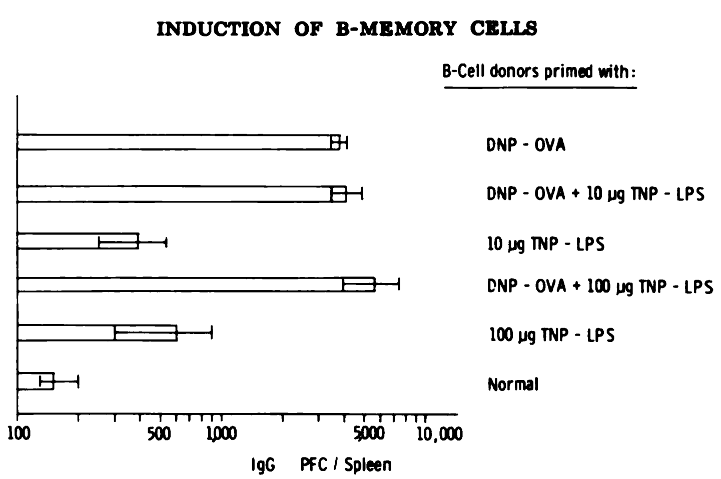

FIG. 4. Effect of simultaneous injection of TI and TD antigens on B-memory cell induction. Irradiated recipients were repopulated with 8 × 10^6 T cells from mice primed with KLH and 10 × 10^6 B cells from mice primed with the antigens indicated in the figure. Recipient mice were challenged with 10 μg DNP-KLH. Values shown are mean DNP-specific indirect (IgG) PFC/spleen ± SEM (five mice/group).

It is also possible that TI antigens are unable to prime B_γ memory cells because they preferentially activate T_s rather than T_H. These T_s could prevent memory cell priming by suppressing T_H (23) or by directly suppressing the B_γ cell (1, 4). The results presented here suggest, but do not prove, that this is an unlikely explanation, since the induction of B_γ memory cells was not suppressed when B-cell donors were primed simultaneously with a TI and a TD antigen (Fig. 4). Moreover since T cells were eliminated from the DNP (TNP)-primed B cells before transfer, T_s could not have prevented the expression of memory in this system. It should be emphasized, however, that T_s have not been shown to markedly influence the antibody response to the two TI antigens used here, TNP-LPS and DNP-Ficoll (24). Possibly other TI antigens such as S3 or polyvinylpyrrolidone which are known to activate T_s (13, 25, 26) would suppress B_γ memory cells induced by a TD form of those antigens. Studies are in progress to investigate this possibility by using the S3 antigen.

Thus, TI antigens are presumably unable to activate T_H and/or B_γ precursor cells which are required for the induction of IgG memory responses. Although the present results do not provide any information as to which of these two cell types fails to be activated by TI antigens, it is known from other studies that TI antigens do have the ability to activate B_γ cells. For example, S3 can elicit good IgG responses when T_H are nonspecifically activated by allogeneic cells (27), when B-memory cells are induced by a TD form of the antigen, i.e. S3 coupled to erythrocytes (S3-RBC) (19, 28) or when T_s are eliminated (4, 27). Moreover at least some preparations of DNP-Ficoll elicit significant IgG responses (8) particularly when T_H are nonspecifically activated by allogeneic cells (29). However in none of these cases will a second injection of these antigens elicit an IgG memory response (8 and H. Braley-Mullen, unpublished results).

Since TI antigens do have the ability to activate B_γ cells the inability of TI antigens to prime B_γ memory cells is more likely to be due to the fact that TI antigens cannot activate T_H. We and others have previously shown that T_H are required to induce differentiation of B_γ cell precursors to memory cells after priming with TD antigens (10–12) although the evidence on this point is controversial (14–16). Moreover there is no evidence as yet that any TI antigen can directly activate conventional antigen-specific T_H. If T_H could be activated by a TI antigen under an appropriate experimental condition we would predict that such an immunization procedure should result in the induction of B_γ memory cells for that antigen. Studies are now in progress to test this hypothesis.

Summary

Mice were primed with dinitrophenyl (DNP) (trinitrophenyl, TNP) coupled to thymus-independent (TI) or thymus-dependent (TD) carriers. B cells from these mice were transferred to irradiated recipients with T cells from keyhole limpet hemocyanin (KLH)-primed mice. After secondary immunization with DNP-KLH a significant DNP-specific IgG memory response was produced only by mice which received B cells which had been primed with TD antigens. TI antigens were unable to induce differentiation of B-cell precursors to IgG producing memory B cells but they did not suppress the induction of B-memory cells by TD antigens. The results indicate that TI antigens fail to activate a cell type which is required for the induction of memory B cells.

The author thanks Patra Mierzwa and Catherine Dunn for skilled technical assistance, Vicky Spate of the University of Missouri Research Reactor for irradiating the mice, and Kathleen Schallert, Linda O'Connor, and Debbie Harman for typing the manuscript.

Received for publication 28 December 1977.

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Brief Definitive Report

SECRETORY COMPONENT ON EPITHELIAL CELLS IS A SURFACE RECEPTOR FOR POLYMERIC IMMUNOGLOBULINS*

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The transport of immunoglobulins across mucous membranes is a selective process that involves different classes of immunoglobulins in different species (1). In human external secretions, the principal immunoglobulin is secretory IgA (s-IgA). This molecule is the product of plasma cells that secrete polymeric IgA with J chain, and of epithelial cells that synthesize secretory component (SC). The SC becomes linked to the IgA during transport through the epithelial cells (2). In some cases of IgA deficiency, secretory IgM (s-IgM) rather than s-IgA appears in external secretions (2, 3). Several models of active transport proposed to explain the selective appearance of IgA and IgM in secretions suggest that initially these immunoglobulins are selectively bound to a receptor on the surface of exocrine epithelial cells (2, 4). The possibility that SC may serve as an immunoglobulin receptor on epithelial cells is implied by the following findings: J chain-containing polymeric IgA and IgM molecules exhibit an in vitro affinity for SC (2); the infusion of an animal with anti-SC serum lowers the ratio of intestinal s-IgA to serum IgA (5); and immunofluorescence studies on tissue sections of the intestine reveal the coincidental appearance of SC and IgA on the basal and lateral surfaces of epithelial cells (2).

The present studies were undertaken to determine whether SC is indeed a receptor for immunoglobulins of various classes, subclasses, and molecular configurations. The human epithelial cell line HT-29, which displays SC on the cell surface (6), and sections of human fetal intestines were examined for their ability to bind various immunoglobulins on the cell membrane or in the cytoplasm.

Materials and Methods

Preparation of Immunoglobulins and SC. Secretory IgA from colostrum, and polymeric or monomeric IgA from sera of patients with IgA myelomatosis, and IgG from normal human serum were purified by ammonium sulfate precipitation, followed by molecular sieve chromatography on Sephadex G-200 and Sepharose 6B, and DEAE-cellulose chromatography as described in detail (7). IgM proteins were purified from sera of patients with Waldenström's macroglobulinemia by euglobulin precipitation with a 15-fold excess of cold distilled water and subsequent Sephadex G-200 gel filtration (8). SC was purified by the method of Brandtzaeg (9) from colostrum and milk of normal mothers and a patient with panhypogammaglobulinemia. The purity of these preparations was determined by immunoelectrophoresis and double immunodiffusion with polyvalent

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* Supported by U.S. Public Health Service Research grants GM-07164, AI-10854, DE-02670, and CA-13148.

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antisera to human serum and colostrum (Behring Diagnostics, American Hoechst Corp., Sommerville, N.J.).

Reagents. Monovalent antisera (specific for the heavy (H) chains of IgA, IgM, or IgG) were prepared in this laboratory as previously described (7), or were obtained from a commercial source (Behring Diagnostics). Anti-SC reagents were prepared by adsorption of anti-s-IgA antisera with purified serum IgA (10) or by immunization of rabbits and goats with SC that was purified as described above; a commercially available rabbit anti-human SC was also used (Behring Diagnostics). Preparations of anti-J chain and anti-lactoferrin reagents have been described (7, 11). Procedures used for the preparation and fluorochrome labeling of γ-globulin fractions and $F(ab')_2$ fragments of anti-SC and anti-H chain reagents have been described (12).

Tissue and Cell Processing. Tissue from the small intestine was obtained from human fetuses (age determined by crown to rump measurements). Tissues were either immediately frozen in liquid nitrogen or were fixed in cold 95% ethanol and embedded in paraffin by the method of Sainte-Marie (13); frozen sections (4 μm) were fixed in 95 ml of 95% ethanol + 5 ml of glacial acetic acid. The presence of SC was determined by incubation with tetramethylrhodamine-isothiocyanate (TRITC) labeled anti-SC. To determine the binding of immunoglobulins, tissue sections were incubated with TRITC-labeled monomeric or polymeric myeloma IgA, colostral IgA, macroglobulin IgM, or IgG.

A line of human epithelial cells, HT-29 (developed at the Sloan-Kettering Institute for Cancer Research, New York) was propagated in tissue culture and allowed to grow in monolayers, according to the technique of Huang et al. (6). In some experiments, cells were recovered from monolayers by a 5-min treatment at 37°C with 0.25% trypsin and 10^{-2} M EDTA, washed, and then allowed to adhere to sterile glass cover slips for 48–72 h before examination. In other experiments, cells were scraped off the flask wall, washed, and resuspended in phosphate-buffered saline (PBS) + 1% bovine serum albumin + 0.3% sodium azide for immediate examination. Cultures were maintained in McCoy's Modified 5A medium (Grand Island Biological Co., Grand Island, N. Y.) that contained 15% fetal bovine serum (Reheis Chemical Co., Kankakee, Ill.), penicillin and streptomycin, and L-glutamine. All cultures were incubated at 37°C in 5% CO_2.

The presence of SC on the cell membrane was determined with the fluorochrome-labeled $F(ab')_2$ fragment of anti-SC reagents. Binding affinities were determined by incubation of cells with various immunoglobulin preparations (50 μl of a solution containing 4 mg/ml) for 45 min at room temperature. Cells were washed in PBS and then incubated with fluorochrome-labeled anti-H chain reagents or their $F(ab')_2$ fragments.

Cell suspensions were prepared as described by Winchester (12), and all specimens were examined with a Leitz Orthoplan (Leitz, Wetzlar, Germany) fluorescence microscope equipped with epi-illumination.

Results

Immunoglobulin Binding by Intestinal Tissues. Sections of human small intestine obtained from fetuses at 8, 10, 11, 12, 13½, 14, and 16 wk of gestation were examined by direct immunofluorescence for the presence of SC and endogenous immunoglobulins. Intestinal tissue sections incubated with labeled anti-H chain reagents alone exhibited no cellular fluorescence, which indicated that endogenous immunoglobulins were absent. SC was not detected in fetal tissues until the 16th-wk of gestation. These findings match those of Ogra et al. (14) as to the time of appearance of SC in human fetal development. At this stage, the epithelial cells fluoresced on staining with fluorochrome-labeled polymeric IgA (Fig. 1a), whereas epithelial cells of tissue sections from younger fetuses (which displayed no SC) did not exhibit immunoglobulin binding. Specificity of the immunoglobulin binding (at 16 wk) was confirmed by the absence of fluorescence when the cells were pre-incubated with unlabeled polymeric IgA. TRITC-labeled monomeric IgA and serum IgG did not bind to the cytoplasm of epithelial cells from fetuses of any age. The fluorescence observed after incubation with fluorochrome-labeled IgM was weaker than with poly-

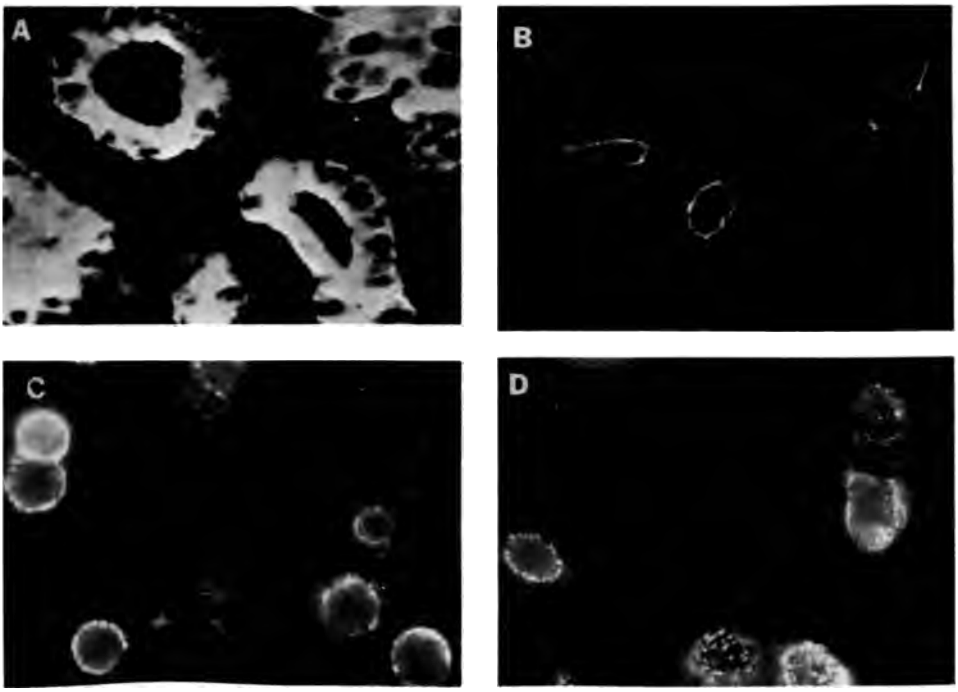

FIG. 1a. Binding of RITC-labeled polymeric IgA to the cytoplasm of fetal intestinal epithelium at 16 wk of gestation; b. Expression of SC on the surface of HT-29 colon carcinoma cells as disclosed by RITC-labeled anti-SC reagent; c. Binding of polymeric IgA to the surface of HT-29 cells; d. Binding of IgM to the surface of HT-29 cells.

meric IgA; fluorescence of marginal intensity was observed with TRITC-labeled s-IgA.

Binding of Immunoglobulins to the Surface of Colonic Carcinoma Cells. HT-29 cells attached to a cover slip or in single cell suspensions were examined for the presence of SC on the membrane and for their ability to bind immunoglobulins of various classes, subclasses, and molecular configurations. SC was expressed most prominently after 72 h of cultivation; at that time 35% of the cells fluoresced with labeled anti-SC reagents (Fig. 1b). Incubation of these cells with polymeric IgA myeloma proteins and subsequent addition of fluorescein isothiocyanate-labeled alpha chain specific antisera revealed immunoglobulin binding to the cell surface. Both IgA1 and IgA2 subclasses with kappa and lambda chains were included in these IgA myeloma proteins. Characteristics of these immunoglobulins and their capacities for binding to HT-29 cells are given in Table I and Fig. 1c. Binding of immunoglobulins to epithelial cells was not affected by IgA subclass or light (L) chain type, but was dependent on molecular configuration, as shown by the failure of monomeric IgA and s-IgA to bind to HT-29 cells. Three of four IgM proteins also bound to the surface of HT-29 cells. The properties of the immunoglobulins are indicated in Table I and Fig. 1d. IgG or labeled anti-H chain reagents did not react with the cells.

The binding of polymeric IgA and IgM to the surface of epithelial cells could be blocked by incubating the cells with anti-SC before adding immunoglobulins;

TABLE I

Binding of Immunoglobulins by HT-29 Epithelial Cells

Class	Configuration*	J Chain‡	L Chain	Binding
IgA2	Polymeric	+	λ	+
IgA1	Polymeric	+	κ	+
IgA1	Monomeric	–	κ	–
IgA1	Monomeric	–	κ	–
s-IgA	Polymeric	+	κ and λ	–
IgM	Polymeric	+	κ	+
IgM	Polymeric	+	λ	+
IgM	Polymeric	+	κ	–
IgM	Polymeric	+	κ	+
IgG	Monomeric	–	κ and λ	–

* Configuration determined by polyacylamide gel electrophoresis and ultracentrifugation (26).

‡ Presence of J chain determined by polyacylamide gel electrophoresis and immunoelectrophoresis (26).

anti-lactoferrin or anti-J chain reagents had no blocking effect.

HT-29 cells treated with trypsin lost their membrane-associated SC as evidenced by their failure to react with fluorochrome-labeled anti-SC; these SC-negative cells did not bind monomeric or polymeric immunoblobulins of any class. However, when these treated HT-29 cells were cultivated for at least 24 h, surface SC reappeared and the binding of polymeric immunoglobulins was restored.

Discussion

SC is a glycoprotein constituting an integral part of the s-IgA molecule. A number of conjectures have been put forth to account for its function. Within the s-IgA molecule, SC affords protection against attack by proteolytic enzymes (15) and contributes to the stabilization of the quaternary structure of s-IgA (4, 16, 17). South et al. (18) suggested that SC might be instrumental in the transport of IgA from serum into external secretions. However, the parenteral infusion of large amounts of serum IgA did not increase the levels of IgA in secretions because serum-derived 7S IgA is not transported into external secretions (4). Subsequent investigations revealed that only polymeric, J chain-containing IgA and IgM were transported into exocrine fluids (2, 4).

It has also been postulated that SC might be involved in the homing of IgA precursor cells to secretory tissues. This possibility was prompted by the absence of IgA-containing cells from the submucosal lymphoid tissues of an SC deficient patient (19). However, the failure to reveal SC receptors on the surface of normal or stimulated peripheral blood lymphocytes (20, 21) did not support this contention.

Earlier investigations strongly implied that SC mediates the transport of polymeric immunoglobulins (2, 4, 5), and proposed that SC acts as a specific receptor (2). However, evidence has not been offered for the binding of polymeric immunoglobulins to membrane-associated SC on living epithelial cells. Results of the present study validate the concept that SC does indeed function as an immunoglobulin receptor. We have demonstrated that the binding of polymeric immunoglobulins to the surface or cytoplasm of epithelial cells requires the presence of SC and is inhibited by anti-SC. We have also shown that only polymeric immunoglobulins devoid of SC will bind to intestinal epithelial cells and will do so irrespectively of IgA subclass and L-chain type. The participation of J chain in the process of SC binding of polymeric immunoglobulins remains unresolved (2, 22).

This study concerns only the initial event in the transport of immunoglobulins through the epithelial cells. Reports of IgA associated with SC in cytoplasmic vesicles within epithelial cells (23–25) suggest that membrane-bound IgA is then internalized and transported to the secretory surface of the exocrine cell.

Summary

Epithelial cells of human fetal intestines and of a colonic carcinoma cell line (HT-29) exhibited intracellular and surface binding of polymeric immunoglobulins of IgA and IgM classes; monomeric IgA and IgG did not bind to these cells. Secretory component was identified as the receptor involved in the immunoglobulin binding. This conclusion was confirmed by the following experiments: trypsin abrogated the surface binding of polymeric immunoglobulin, reappearance of surface secretory component (SC) restored immunoglobulin binding; the appearance of SC in developing fetal tissues coincided with their potential to bind polymeric immunoglobulin; anti-SC reagents inhibited the binding of immunoglobulins to epithelial cells; and SC-containing secretory IgA did not bind to the surface of HT-29 cells.

We thank Dr. J. Fogh and M. A. Egan for the donation of the HT-29 cell line and for their advice on tissue culture techniques. We are indebted to Dr. F. W. Kraus and Ms. C. Sims for editorial advice.

Received for publication 21 February 1978.

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Brief Definitive Report

RESTRICTED HELPER FUNCTION OF $F_1 \rightarrow$ PARENT BONE MARROW CHIMERAS CONTROLLED BY *K*-END OF *H-2* COMPLEX

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Although the question of whether T and B lymphocytes collaborate across major histocompatibility complex (MHC) barriers remains controversial (1-5), there is general agreement that F_1 hybrid T cells collaborate with parental strain B cells. This paper presents an exception to this rule. It will be shown that F_1 T cells differentiating from stem cells in mice of one parental strain collaborate well with B cells from this strain, but lose their capacity to stimulate B cells of the opposite strain.

Materials and Methods

Mice. CBA/Cum (CBA, $H\text{-}2^k$), C57BL/6 (B6 $H\text{-}2^b$), and (CBA × B6)F_1 mice were obtained from Cumberland View Farms, Clinton, Tenn. C57BL/10 (B10, $H\text{-}2^b$) and B10.Br ($H\text{-}2^k$) mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. (B10 × B10.Br)F_1 mice were bred in our laboratory. B10.A (4R) mice were a gift from Dr. W. L. Elkins, University of Pennsylvania.

Chimeras. Split-dose irradiation was used to prepare the chimeras. CBA and B6 mice were exposed to 600 rads (6), left for 2 wk, and then given 850 rads. 4 h later the mice received an intravenous injection of 3×10^7 (CBA × B6)F_1 bone marrow cells treated with anti-thy 1.2 antiserum plus complement (3).

Assay for T-B Collaboration. As described in detail elsewhere (6), T cells (0.8×10^6) and B cells ($5\text{-}8 \times 10^6$ anti-thy 1.2-treated spleen cells from mice primed with sheep erythrocytes [SRC] 2 mo before) were transferred with SRC (0.1 ml of 5% solution) into irradiated (750 rads) (CBA × B6)F_1 mice. Direct (IgM) and indirect (IgG) plaque-forming cells (PFC) were then measured in the spleen 7 days later.

Results

Cytotoxic indices with CBA anti-B6 and B6 anti-CBA alloantisera plus complement (6) showed that, for both (CBA × B6)F_1 marrow → irradiated CBA chimeras ($F_1 \rightarrow$ CBA chimeras) and $F_1 \rightarrow$ B6 chimeras, >97% of spleen and lymph node (LN) cells from the chimers were of donor F_1 origin. This applied to 10 of 10 chimeras tested 3-12 mo after marrow reconstitution.

To test the helper function of the chimeras, unprimed T cells prepared from LN were first activated to SRC in irradiated (CBA × B6)F_1 mice; this was to ensure that the first exposure of the F_1 T cells to antigen was in a "normal", i.e. F_1, environment. 4×10^7 nylon-wool-purified LN T cells (>90% thy 1.2-positive) (7) from $F_1 \rightarrow$ CBA chimeras were transferred intravenously with SRC (0.5 ml

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[*] Supported by grants AI-10961 and CA-15822 from the U. S. Public Health Service.

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TABLE I

Restricted Helper Function of (CBA × B6)F_1 → CBA Bone Marrow Chimeras Linked to H-2 Complex

T-cell group	Donor of helper T cells[*]	B cells[‡]	Anti-SRC PFC/spleen at 7 days in irradiated (CBA × B6)F_1 mice	
			IgM	IgG
1	F_1 → CBA chimeras	B10.Br	62,120 (1.11)[§]	123,250 (1.29)
		B10	0	0
		(B10 × B10.Br)F_1	75,600 (1.16)	200,940 (1.05)
2	Normal (CBA × B6)F_1	B10.Br	47,290 (1.10)	126,000 (1.29)
		B10	35,540 (1.38)	70,750 (1.24)
		(B10 × B10.Br)F_1	95,250 (1.38)	226,120 (1.24)
Groups 1 + 2 (0.8 × 10^6 of each)		B10	29,250 (1.21)	64,940 (1.08)

[*] Unprimed T cells pooled from five chimeras reconstituted with marrow 1 yr previously were activated to SRC in irradiated normal (CBA × B6)F_1 mice before use as helper cells (see text); activated helper cells were recovered from thoracic duct lymph of the recipients at 5 days post-transfer.

[‡] Anti-thy 1.2-serum-treated spleen cells from SRC-primed mice were transferred intravenously in a dose of 5 × 10^6 viable cells (8 × 10^6 for B10 B cells) with T cells (0.8 × 10^6) and SRC (0.1 ml of 5%) into irradiated (750 rads 1 day before) (CBA × B6)F_1 mice.

[§] Geometric mean of data from four mice per group. Values in parentheses refer to the numbers by which the means are multiplied or divided to give upper and lower limits, respectively, of SE. Background values obtained when B cells were transferred without T cells have been subtracted. These values (PFC/spleen) were: B10.Br 440(1.48) (IgM), 1,630(1.05) (IgG); B10 670(1.24) (IgM), 980(1.27) (IgG); (B10 × B10.Br)F_1 2,580(1.30) (IgM), 4,670(1.79) (IgG). Numbers of PFC when T cells were transferred without B cells were < 100 PFC/spleen.

of 25% solution) into irradiated (800 rads 1 day before) normal (CBA × B6)F_1 mice; control groups of these mice received T cells from normal (CBA × B6)F_1 mice plus SRC. Donor cells were recovered from thoracic duct lymph of both groups of recipients 5 days later (6).

As shown in Table I, SRC-activated (CBA × B6)F_1 T cells derived from F_1 → CBA chimeras gave high IgM and IgG anti-SRC PFC responses with B10.Br (H-2^k) B cells, but gave no response with B10 (H-2^b) B cells. This did not seem to be the result of suppression, since a mixture of chimera F_1 T cells and normal F_1 T cells gave good responses with B10 B cells. Both groups of T cells collaborated well with (B10 × B10.Br)F_1 B cells.

Table II shows that the restriction in helper function was reversed when (CBA × B6)F_1 T cells were derived from F_1 → B6 chimeras, i.e. good collaboration occurred with B10 B cells, whereas only a poor response was seen with B10.Br B cells (the latter response was significant but represented <8% of the response observed when B10.Br B cells were transferred with F_1 → CBA chimera T cells). The restriction mapped to the *K* end of the *H-2* complex since B cells from B10.A(4R) mice (K^kI-A^kI-B^b- - -D^b) were stimulated by T cells from F_1 → CBA chimeras, but not by T cells from F_1 → B6 chimeras.

Discussion

Previous work has shown that although homozygous T cells from normal (nonchimeric mice) fail to collaborate with *H-2*-different B cells in vivo (2, 5), T cells taken from tetraparental bone marrow chimeras stimulate B cells derived from either of the two parental strains involved (3). To explain this discrepancy Katz et al. (2) suggested that T cells differentiating from stem cells in an *H-2*-different environment develop abnormal "cell-interaction determinants", enabling these cells to stimulate B cells of the opposite parental strain. This "adaptive differentiation" hypothesis has recently been refined by Zinkernagel

TABLE II

Helper Function of T Cells from $F_1 \rightarrow$ CBA and $F_1 \rightarrow$ B6 Chimeras Controlled by K-End of H-2 Complex

T-cell group	Donor of helper T cells*	B cells‡	*H-2* region of B cells					Anti-SRC PFC/spleen at 7 days in irradiated (CBA × B6)F_1 mice	
			K	*I-A*	*I-B*	---	*D*	IgM	IgG
		B10.Br	k	k	k	---	k	79,090 (1.04)§	134,300 (1.12)
1	$F_1 \rightarrow$ CBA chimeras	B10	b	b	b	---	b	470 (2.50)‖	200 (1.97)‖
		B10.A(4R)	k	k	| b	---	b	25,260 (1.10)	55,605 (1.15)
		B10.Br	k	k	k	---	k	2,970 (1.07)	9,870 (1.21)
2	$F_1 \rightarrow$ B6 chimeras	B10	b	b	b	---	b	32,030 (1.11)	50,520 (1.18)
		B10.A(4R)	k	k	| b	---	b	0	0
		B10.Br	k	k	k	---	k	53,310 (1.32)	126,500 (1.33)
3	Normal (CBA × B6)F_1	B10	b	b	b	---	b	27,720 (1.20)	42,530 (1.23)
		B10.A(4R)	k	k	| b	---	b	10,770 (1.09)	42,320 (1.23)
Groups 1 + 2		B10.Br	k	k	k	---	k	86,400 (1.28)	134,610 (1.26)
Groups 1 + 2		B10	b	b	b	---	b	31,060 (1.22)	46,250 (1.40)

* Unprimed T cells pooled from three chimeras per group activated to SRC for 5 days in irradiated (CBA × B6)F_1 mice as for Table I. The donor $F_1 \rightarrow$ CBA chimeras and $F_1 \rightarrow$ B6 chimeras were reconstituted with marrow 1 yr and 3 mo previously, respectively.

‡ As for Table I.

§ As for Table I. Background numbers of PFC obtained when T cells were transferred without T cells were: B10.Br 1,810(1.29) (IgM), 10,320(1.03) (IgG); B10 950(1.13) (IgM), 1,330(1.35) (IgG); B10.A(4R) 1,140(1.69) (IgM), 1,190(1.18) (IgG). PFC numbers for T cells transferred without T cells all < 200 PFC/spleen.

‖ Not significantly above values of B cells transferred without T cells ($P > 0.05$).

et al. (8, 9). These workers observed that for T-cell-mediated lympholysis (CML) of virus-infected target cells, F_1 T cells from (a × b)$F_1 \rightarrow a$ chimeras lysed target cells from strain a and (a × b)F_1, but did not lyse strain b targets. From this and other evidence it was concluded that CML occurred only with targets which shared *H-2* determinants with the thymus in which the T cells differentiated from stem cells.

The data in the present paper are consistent with this hypothesis and suggest that the thymus controls the specificity of not only T cells responsible for CML, but also of T helper cells involved inT-B collaboration. It should be mentioned that although there is clear evidence that the thymus per se rather than other microenvironments controls T-cell specificity for CML (9), this has yet to be proved for T-helper function.

Recent studies in this laboratory have suggested that T cells from normal (a × b)F_1 mice behave functionally as a 50:50 mixture of (mutually tolerant) T cells derived from the two parental strains; each subgroup of T cells appears to be able to collaborate with B cells derived from only one of the two parental strains (6, 10). By analogy with the data of Zinkernagel et al., one can suggest that these two subgroups of T helper cells are generated as the result of their stem cell precursors encountering *H-2* determinants of both strain a and strain b on thymic epithelial cells during early differentiation. The progeny of these T-cell precursors then collaborate in a restricted fashion with B cells of strain a and b, respectively. A prediction from this notion which is confirmed in the present paper, is that when (a × b)F_1 T cells differentiate from stem cells in strain a mice, only one of the two subgroups of T cells is generated, namely the subgroup able to collaborate with B cells from strain a.

A further prediction is that homozygous T cells of strain a differentiating from stem cells in (a × b)F_1 mice should resemble normal (a × b)F_1 T cells in function. One subgroup of cells should collaborate with syngeneic (strain *a*) B cells, but not with allogeneic (strain *b*) B cells; the other subgroup should stimulate only allogeneic and not syngeneic B cells. Preliminary studies on the helper function of parent → F_1 chimera T cells activated to SRC in irradiated parental strain mice support this prediction (J. Sprent, unpublished data).

Summary

F_1 → parent bone marrow chimeras were prepared by transferring F_1 hybrid marrow cells into heavily irradiated parental strain mice. When unprimed, donor-derived F_1 T cells from the chimeras were activated to sheep erythrocytes (SRC) for 5 days in irradiated normal F_1 mice, high IgM and IgG anti-SRC responses were observed with F_1 B cells, and with B cells *H-2*-compatible with the strain in which the T cells were raised from stem cells. Significantly, however, responses with B cells of the opposite parental strain were either absent or very low. The restriction in T-helper function mapped to the *K*-end of the *H-2* complex and could not be attributed to active suppression.

Stimulating discussion with D. B. Wilson and the skillful typing of Miss K. Nowell are gratefully acknowledged.

Received for publication 27 February 1978.

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Brief Definitive Report

HEMATOPOIETIC THYMOCYTE PRECURSORS:

IV. Enrichment of the Precursors and Evidence for Heterogeneity*

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The hematopoietic precursor of thymocytes (prothymocyte) is a specialized cell, committed to thymocyte differentiation and discrete from both the already characterized precursors of B cells and the pluripotential stem cell (1–3). We have developed a quantitative assay for this cell and have previously used it to explore the kinetics of repopulation of the murine thymus after γ-irradiation (4) and to establish that prothymocytes are found among the null cell population of both the spleen and bone marrow. They may be distinguished from other cells in this population since they bear surface antigens reactive with rabbit anti-mouse brain antisera and can be induced to display characteristic thymocyte differentiation antigens (5, 6). The physical and surface properties of these cells have permitted us to obtain populations which are enriched 40-fold. Preparations of precursors from bone marrow contain the enzyme terminal deoxyribonucleotidyl transferase (Tdt) while these from spleen lack this activity.

Materials and Methods

Mice – Source and Care. Female mice, 5–6 wk old, were obtained from The Jackson Laboratory, Bar Harbor, Maine. (hereafter designated as AKR/J) and Cumberland View Farms, Clinton, Tenn. (AKR/Cumberland, hereafter designated as AKR/C). AKR/J mice bear the Thy 1.1 (θAKR) alloantigen, while AKR/C anomalously bear Thy 1.2 (θC$_3$H) (7).

Antisera and Complement. Anti-Thy 1 antisera were produced by the reciprocal immunization of AKR/J and AKR/C mice with thymocytes. The preparation of these sera as well as rabbit antisera to mouse brain and mouse Ig has been described previously (4).

Rabbit serum selected for low toxicity to mouse thymocytes was used as a source of complement in alloantiserum-mediated cytotoxicity; guinea pig serum was uced as a complement source in heteroantiserum-mediated killing.

Origin of Cells Repopulating the Thymus. The assay for cells capable of repopulating the thymuses of irradiated mice has been described in detail (2). Thymocytes derived from the injected cells are identified by a cytotoxic test making use of the difference in Thy 1 antigens in AKR mice purchased from different sources. AKR/C (Thy 1.2) mice are injected with hematopoietic cells from AKR/J (Thy 1.1) mice. The proportion of cells in the regenerating thymus which bear the donor type Thy 1 antigen is determined by selectively killing the thymocytes bearing one of the two Thy 1 alleles and measuring residual [^{3}H]TdR incorporation.

To facilitate comparison of the activity of the various populations tested, their repopulation

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* Supported by U. S. Public Health Service grants AI 11326 and HD 09130 and contract NCI CB 43927.

‡ Recipient of Career Development Award HD 39968.

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activity was measured. This was defined as the number of cells required to produce 10% repopulation of AKR/C thymus with ALR/J derived thymocytes 14 days after transfer and was calculated by dividing the number of cells injected by the percentage of cells of donor origin found (× 10).

Cell Preparation. The procedure for inducing the appearance of thymic alloantigens with thymopoietin and the techniques for separating cells on discontinuous gradients of bovine serum albumin (BSA), eliminating Thy 1 or mouse immunoglobulin-positive cells and depleting complement receptor-jpositive lymphocytes have all been described in detail (2, 6, 8).

Terminal Deoxyribonucleotidyl Transferase. (EC 2.7.7.31 deoxyribonucleoside triphosphate: DNA nucleotidyl exotransferase; terminal transferase) was assayed in preparations after homogenization and partial purification by phosphocellulose chromatography as described by Kung et al. (8), with 2.5 μg oligo (dA 12-18) obtained from P-L Biochemicals, Inc., Milwaukee, Wis. as an initiator. The polymerization was followed by measuring the incorporation of [^{3}H]dG (supplied as the triphosphate, sp act 12 Ci/mM) into trichloroacetic precipitable material. [^{3}H]dGTP was purchased from New England Nuclear Corp., Boston, Mass. 1 U of enzyme activity is the amount which catalyzes the incorporation of 1 nmol of deoxynucleotide monophosphate into acid insoluble material in 1 h.

The soluble material obtained between 4×10^7 and 10^9 nucleated cells was applied to each phosphocellulose column. The activity in each cell population was calculated by integrating the activity found in all enzymatically active fractions. The two peaks of activity described by Kung et al. were not calculated separately.

Results

Isolation of Prothymocytes. Spleen cells or bone marrow cells, suspended in RPMI-1640 and 7 mg/ml BSA are initially separated by flotation on either a single step BSA or Ficoll-Hypaque density gradient. The least dense third of the initial spleen cell preparation is used for the subsequent purification. The population accumulating at the interface is removed, suspended at a concentration of 10^7/ml, and incubated sequentially with rabbit antiserum to mouse immunoglobulin and guinea pig complement, and then anti-Thy 1 antiserum and rabbit complement. The cells are then pelleted by centrifugation and resuspended in warm (37°C) isotonic Tris-buffered ammonium chloride (ACT). This step was originally introduced to remove erythrocytes (10). We find that it consistently removes a population of nucleated cells and increases the efficiency of the next step in removing dead or damaged cells. After 10 min in ACT the cells are diluted five-fold with cold (4°C) medium and passed through a glass wool column. The eluted cells are again pelleted and resuspended at 10^7/ml. Complement receptor (CR) positive cells which escaped the anti-Ig treatment are removed and a further separation on the basis of density achieved by flotation in a discontinuous gradient of BSA. The cells accumulating at the 25 and 27% interfaces are pooled, washed, and tested for their prothymocyte activity.

Examples of the results achieved by using this procedure are shown in Table I. Also included are the proportion of cells in each fraction which become Thy 1 positive after thymopoietin treatment. The degree of enrichment of the inducible cells directly paralleled that of the thymus-repopulating activity. The starting materials used here were suspensions of cells from 10 wk old female AKR/J mice. The results are representative of many other experiments with young adult mice. The degree of enrichment achieved does vary with the age of the mice used for the experiments and the greatest repopulation activity was obtained when spleen cells from weanling mice were used (0.08×10^6 cells required to achieve 10% repopulation after 14 days).

TABLE I

Enrichment of Prothymocytes

Fraction		Recovery* of cells	Repopulation‡ activity	Degree of enrichment	Tp§ inducible
					%
S-I	Unfractionated spleen	5.0×10^8	7.0×10^6	1	ND‖
S-II	Hypaque-Ficoll	1.5×10^8	3.4×10^6	2.1	ND
S-III	Anti-Ig + Anti-Thy 1	4.0×10^7	1.1×10^6	6.4	(1)¶
S-IV	ACT Treated	2.6×10^7	0.81×10^6	8.8	(5)
S-V	Glass wool filtered	1.0×10^7	0.39×10^6	17.5	15
S-VI	CRL Depleted‖ BSA Gradient	2.3×10^6	0.23×10^6	30.4	27
M-I	Unfractionated BM	1.8×10^8	1.20×10^6	1	ND
M-II	Hypaque-Ficoll	9.2×10^7	0.66×10^6	1.8	ND
M-III	Anti-Ig and anti-Thy 1	4.8×10^7	0.58×10^6	2.1	(6)
M-IV	ACT Treated	4.1×10^7	0.52×10^6	2.3	11
M-V	Glass wool filtered	1.3×10^7	0.25×10^6	4.8	21
M-VI	CRL Depleted BSA Gradient	4.0×10^6	0.11×10^6	10.9	46

* Viable cells corrected for sampling losses.

‡ Number of cells required to produce 10% repopulation of AKR/C thymus with AKR/T derived thymocytes 14 days after transfer.

§ The percentage of cells rendered susceptible to α-Thy 1.1 antiserum by incubation with thymopoietin. This is calculated by subtracting the percentage killed by this serum in a control sample of cells (not treated with thymopoietin) from the percentage of cells killed in a thymopoietin-treated sample. A total of 200 cells was counted for each determination. The results shown are the average of duplicates. In similar experiments the coefficient of variation for the determination of inducible cells is 22%. Due to the small number of inducible cells present no attempt was made to estimate these cells until the Thy 1.1-positive cells present in the bond marrow and spleen cell preparations had been eliminated by treatment with anti-Thy 1.2 serum.

‖ CRL, complement receptor lymphocyte; ND, not done.

¶ Because of the statistical problems inherent in counting small numbers of cells we regard only values greater than 10% as significant. The parentheses indicate results which do not meet this criterion.

Terminal Deoxyribonucleotidyl Transferase in Prothymocyte Enriched Fractions. Bollum (10) has described an enzyme with the unusual property of adding deoxyribonucleotides to a DNA primer in the absence of a template. The principal source is the thymus, where it is found in cortical thymocytes (12, 13). Peripheral T cells lack the enzyme. Low concentrations are found in bone marrow (14, 9). No other tissue has detectable activity. Although no function is known for this enzyme, Baltimore (14) has pointed out that it is a potential somatic mutator and could thus be implicated in the generation of immunologic diversity.

Table II shows that the population of bone marrow cells obtained as a consequence of preparing fractions enriched in prothymocytes is substantially (12-fold) enriched in terminal transferase. The degree of enrichment is proportional to the extent of the purification of the precursor cells. This prothymocyte-rich fraction consists of Thy 1-negative cells. The majority of the Tdt-containing cells in the fractions are killed by rabbit antiserum against mouse brain (Table II, line 3). We have previously shown this serum to be cytolytic for thymocyte precursors (2). In three separate experiments with unfractionated bone marrow the reduction in the specific enzymatic activity of the surviving cells varied from 68 to 83%. Neither increasing the antibody concentrations nor subjecting the surviving cells to a second antibody treatment eliminated the residual activity.

No transferase was found in any preparation from spleen cells including one 40-fold enriched in prothymocyte activity (Table II, line 5), nor was any activity detectable in 18-day fetal liver or spleen (data not shown). Mixing spleen cells

TABLE II

Terminal Deoxyribonucleotidyl Transferase Activity of Prothymocyte Fractions

Fraction	Activity
	units/10^8 cells
Untreated bone marrow	0.16
Prothymocyte-rich fraction*	1.91
Anti-mouse brain treated‡ prothymocyte-rich fraction	0.35
Normal rabbit serum treated§ prothymocyte-rich fraction	1.78
Prothymocyte-rich fraction‖ from spleen	0.01

* Cells obtained from a BSA gradient after the prior removal of both glass wool adherent Thy 1.1 and Ig-bearing cells. 3.6 × 10^8 bone marrow cells from 105 AKR/J mice were fractionated to yield 1.6 × 10^8 cells in the prothymocyte-enriched fraction. (Equivalent to fraction M-VI in Table I).

‡ Cells from the prothymocyte-rich fraction treated with rabbit anti-mouse brain and guinea pig complement. Dead cells were removed by centrifugation over Hypaque-Ficoll. Live cells, which accumulated at the interface were washed twice before homogenization.

§ Cells from the prothymocyte-rich fraction treated with normal rabbit serum and guinea pig complement and prepared as above.

‖ Cells obtained from a BSA gradient after the removal of glass wool adherent, Thy 1.1, and Ig bearing cells. 8.3 × 10^8 spleen cells were fractionated to yield 4.3 × 10^7 cells in the prothymocyte-enriched fraction. This fraction had a thymic precursor activity 37 times that of the starting population. (Equivalent to fraction S-VI in Table I).

with thymocytes did not interfere significantly with the assay of the enzyme in the thymocytes.

Discussion

The procedures described here permit the partial purification of the cells responsible for the repopulation of the thymus after irradiation. They are based on the facts that the precursors are relatively low density cells, nonadherent to glass wool, and resistant to the cytotoxic effects of anti-Thy 1 and anti-Ig antisera (2). The entire sequence requires about 4 h and results in a 30-40-fold enrichment of the precursors from spleen. The procedure can also be applied to bone marrow cells but only a 10-15-fold enrichment is obtained with these cells. As expected, both precursor cells and thymopoietin-sensitive cells are purified together. Previous work had indicated that thymopoietin inducible cells can serve as thymocyte precursors (16). The lesser degree of purification achieved with bone marrow is a consequence of both the paucity of Ig-positive and Thy 1-positive cells and the greater number of low density cells in the marrow.

The procedures which lead to the enrichment of the bone marrow precursor also lead to the partial purification of cells which contain the enzyme deoxyribonucleotidyl transferase. This enzyme is present in high concentrations in thymic lymphocytes and it is reasonable to assume that it is also present in the precursor cells. Such an association is also suggested by the susceptibility of both Tdt containing cells and prothymocytes to cytolysis by antisera against mouse brain. Tdt has also been found in the precursor cells which display thymic alloantigens after treatment with thymopoietin or endotoxin (17). Although this is strong evidence that terminal transferase is associated with a prothymocyte in the bone marrow, difficult and intriguing questions remain unanswered. If the enzyme is present in prothymocytes, why can no enzyme be found in populations obtained from spleen or fetal liver? The splenic precursor is otherwise indistinguishable from the bone marrow-derived cell and the assay for transferase is sufficiently sensitive to detect 1% of the amount found in bone marrow. Furthermore, irradiated mice repopulated with Tdt-negative spleen cells have Tdt-positive thymocytes. R. S. Basch, A. V. Hoffbrand, and K. Ganeshaguru, unpublished results.

We have considered a variety of possibilities to explain this apparent heterogeneity. In the simplest, the assumption is made that the precursors only express the enzyme in the bone marrow environment. Either the induction of previously negative cells by the marrow environment, or the accumulation of enzyme-positive cells by that environment, would account for the apparent heterogeneity. In either case we would argue that the precursors are Tdt negative for most of their existence and only acquire the enzyme in the last stages of their prethymic development.

The distribution could also result from a negative influence exerted in the spleen, suppressing enzyme expression in cells which would otherwise contain it. The splenic environment of normal mice does appear to be inimical to the expression of the thymus leukemia (TL) antigen which, like Tdt, is essentially restricted to thymocytes (18).

More complicated models are required if the heterogeneity in Tdt content actually reflects specialization of the precursor pool. It is plausible that some of the diverse T-cell subsets could arise from progenitors who differed from each other in the time at which they acquired or lost Tdt. Furthermore, some Tdt containing bone marrow cells cannot be induced to display thymic alloantigens (17) and are thus presumably not in the T-cell lineage. If terminal transferase-mediated somatic mutation is to be considered as a source of immunologic diversity, the enzyme should appear at some stage of the development of B-cell progenitors. Perhaps these are the other Tdt containing cells of the bone marrow.

Summary

A method has been developed for the enrichment of the hematopoietic precursors of thymocytes from spleen and bone marrow cells. Up to 40-fold enrichments were obtained resulting in preparations in which as few as 10^5 cells produced prompt repopulation of the thymus of an irradiated mouse.

Precursor cells from bone marrow appear to contain the enzyme terminal deoxyribonucleotidyl transferase (Tdt), an agent suggested as a potential somatic mutator. This enzyme (Tdt) was not detectable in any spleen cell preparation examined, including one in which a 40-fold enrichment of thymocyte precursors had been produced. This is the first difference reported between the splenic and bone marrow precursors of thymocytes.

Discussions with Doctors Allen Silverstone and David Baltimore have been invaluable. The excellent technical assistance of Bohumila Fahmy, Thomas Panagiotatos, and Shirley Carchi are gratefully acknowledged.

Received for publication 21 February 1978.

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Brief Definitive Report

THE IMMUNE RESPONSE OF ALLOPHENIC MICE TO 2,4-DINITROPHENYL (DNP)-BOVINE GAMMA GLOBULIN

I. Allotype Analysis of Anti-DNP Antibody*

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The question of whether or not lymphoid cells can cooperate across a histocompatibility difference barrier has been studied in several laboratories. Using an adoptive transfer system, Katz et al. (1) first showed that T cells from (low responder × high responder) F_1 mice, primed to the terpolymer L-glutamic acid, L-lysine, L-tyrosine (GLT), could collaborate with 2,4-dinitrophenyl (DNP)-primed B cells from a high responder, but not a low responder strain, in response to DNP-GLT. The response to GLT is under *H-2*-linked Ir gene control. In contrast, studies with mouse bone marrow chimeras have shown that T cells can interact with *H-2*-histoincompatible B cells in response to antigens not under Ir gene control (2-4). Another type of chimera, the allophenic mouse, has been used to study possible histoincompatible cell interactions to a number of antigens, including DNP-L-glutamic acid, L-lysine, L-alanine; L-glutamic acid, L-alanine, L-tyrosine; L-glutamic acid, L-lysine, L-phenylalanine; and poly-L (Tyr, Glu)-poly D,L-Ala-poly-L-Lys[T,G)-A – L] (5-9). The response to each of these antigens is under *H-2*-linked Ir gene control.

It was initially reported (8, 9) that in allophenic mice containing both high and low responder cells, the antibody to (T,G)-A – L was of both the high and low responder allotype. This was interpreted to mean that high responder T cells had cooperated with low responder B cells across a histocompatibility difference barrier in the environment of the allophenic mice. However, Press and McDevitt (10) have recently reported that additional and more accurate analyses of these allophenic mouse sera failed to detect any anti-(T,G)-A – L antibody of the low responder allotype. Moreover, in an experiment using bone marrow chimeras, there was no low responder allotype antibody produced in response to (T,G)-A – L(10).

The present study was undertaken to test the immune response of allophenic mice to an antigen, DNP-bovine gamma globulin ($DNP_{56}BGG$), known to be controlled by genes both inside and outside the *H-2* complex (11, 12).[1] When high and low responder cells to $DNP_{56}BGG$ are present in allophenic mice, only antibody of the high responder allotype is produced. The results suggest that cell cooperation in allophenic mice cannot occur across a histocompatibility difference barrier in response to an antigen whose genetic control is at least partially within the *H-2* complex.

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* Supported by National Institutes of Health grant AI 11752.

[1] R. C. Newton and C. M. Warner. Manuscript in preparation.

Materials and Methods

Mice. The inbred strains A and C57BL/6 were purchased from The Jackson Laboratory, Bar Harbor, Maine. The F_1 hybrids (A × C57BL/6) were bred in our laboratory. The allophenic mice were produced as previously described (5).

Immunization. The immunization schedule was according to Newton and Warner (11). Mice received 50 μg of $DNP_{56}BGG$ in 1 mg $Al(OH)_3$ (dry weight) administered intraperitoneally on days 1, 29, and 57. The animals were bled, sacrificed, and their spleens were excised on day 64.

Spleen Cell Composition of Allophenic Mice. After spleen excision, a single cell suspension was prepared, the lymphocytes were isolated on a Ficoll-Hypaque density gradient, and the proportion of C57BL/6 parental cells was determined by a trypan blue dye exclusion microcytotoxicity test (6). The anti-C57BL/6 serum was produced by the injection of C57BL/6 spleens into A mice to eliminate any possible cross-reaction with A parental type spleen cells.

Assays for DNP Antibody. Two different radioimmunoassay procedures were used to measure serum antibody levels. The first was a modification of the procedure of Green et al. (13). In this procedure, [^{3}H]DNP-lysine synthesized in our laboratory by the method of Stupp et al. (14) was used as the antigen, and $(NH_4)_2SO_4$ was used to precipitate the antibody-hapten complex (11). The second procedure was according to Freed et al. (12). In this procedure ^{125}I-DNP-bovine serum albumin (BSA), synthesized in our laboratory by the method of Greenwood and Hunter (15), was used as the antigen, and rabbit anti-mouse immunoglobulin (Miles Laboratories Inc., Elkhart, Ind.) was used to precipitate the antibody-antigen complex. Values <5% binding in this assay are insignificant.

Allotype Analysis of DNP Antibody. Antibody to the b allotype (C57BL/6) was prepared according to Herzenberg and Herzenberg (16). Analysis using normal sera and myeloma proteins in double gel diffusion showed that the antiserum was monospecific for the b allotype.

The immunoglobulin fraction was isolated from the anti-allotype serum by ammonium sulfate salt fractionation, and coupled to cyanogen bromide-activated Sepharose 4B by the method of Cuatrecasas (17). A 1-ml column of immunoadsorbent was poured and equilibrated with 0.10 M sodium phosphate buffer, pH 7.0, containing 1.0 mg/ml BSA as a carrier. The sera were assayed for anti-DNP antibody before application to the column. 10 μl of immune sera were applied, and the column was incubated at room temperature for 1 h to ensure complete binding. Then, 5 ml of buffer was passed through the column and collected. This was called the "passed fraction". Elution of the antibody bound to the column was accomplished using 5 ml of 0.10 M acetic acid, pH 3.1, containing 1.0 mg/ml BSA. This fraction, called the "eluted fraction", was neutralized with NaOH to pH 7.0–7.2. Both fractions were then concentrated using a Minicon B-15 (Amicon Corp., Lexington, Mass.) to a final volume of 0.5 ml (a 1:50 dilution of the original serum sample). The passed and eluted fractions were then assayed for anti-DNP antibody activity, as described above.

Results

Table I summarizes data collected in our laboratory which shows that A mice are high responders and C57BL/6 mice are low responders to $DNP_{56}BGG$. Data is also shown for (A × C57BL/6)F_1 hybrid mice.

For the experiments described in this paper, 13 A ↔ C57BL/6 allophenic mice were immunized with $DNP_{56}BGG$. Table II shows that the population of mice included a range of mixtures of parental spleen cell types. The allophenic mice studied were not assessed for total serum allotype, but rather for spleen cell composition to determine their degree of chimerism. However, another study on C57BL/6 ↔ (CBA × CBA/H-T6) allophenic mice (18) has shown that the percentage of each parental cell type is the same when assayed either by spleen cell composition or by total serum allotype composition. The response to

TABLE I

Response of Inbred Strains of Mice to Immunization with $DNP_{56}BGG$

Mouse strain	No. of mice tested	ABC_{33} ± SEM*	Classification
A	26	80 ± 5	High responder
C57BL/6	31	10 ± 2	Low responder
(A × C57BL/6)F_1	10	97 ± 15	High responder

* Data are the reciprocal of the serum dilution for 33% binding of 10^{-8} M [^{3}H]DNP-lysine. ABC_{33}, antigen binding capacity at 33% antigen bound.

TABLE II

Response of A ↔ C57BL/6 Allophenic Mice to Immunization with $DNP_{56}BGG$

Mouse	% C57BL/6 SWBC*	ABC_{33}‡
170	1	32
171	1	88
159	11	128
191	12	100
172	52	100
173	59	65
162	63	43
160	72	26
187	84	0
168	95	33
157	95	3
158	97	9
167	106	47

* Data are the percentage of C57BL/6 spleen white blood cells (SWBC) analyzed by cytotoxicity testing as described in the text. All assays were done in duplicate.

‡ Data are the reciprocal of the serum dilution for 33% binding of 10^{-8} M [^{3}H]DNP-lysine. All assays were done in duplicate. ABC_{33}, antigen binding capacity at 33% antigen bound.

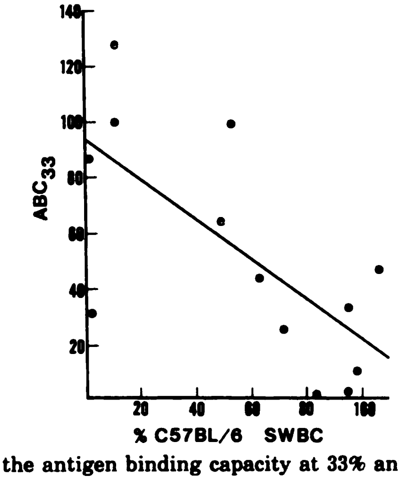

FIG. 1. Correlation of the antigen binding capacity at 33% antigen bound (ABC_{33}) to the percentage of C57BL/6 SWBC. The line is the least squares linear regression through all the data points. Number of mice = 13; $r = -0.68$; $P < 0.01$.

immunization was variable. In general, those mice with a higher proportion of low responder (C57BL/6) spleen cells produced less anti-DNP antibody in response to immunization with $DNP_{56}BGG$. This point is illustrated in Fig. 1.

Five of the mice listed in Table II were chosen for further experimentation. These mice were assayed for the presence of the low responder b (C57BL/6) allotype in the total anti-DNP antibody population, as is shown in Table III. Despite the fact that a significant proportion of C57BL/6 cells were present in

TABLE III

*Allotype Analysis of the Anti-DNP Antibody Produced by Inbred and Allophenic Mice in Response to Immunization with $DNP_{56}BGG$**

Mouse	% Antigen bound before anti-C57BL/6 column	% Antigen bound by passed fraction	% Antigen bound by eluted fraction
A (pooled)	27 ± 4	24 ± 6	2 ± 0
C57BL/6 (pooled)	20 ± 3	6 ± 2	16 ± 6
170	32 ± 3	29 ± 5	4 ± 3
171	37 ± 1	38 ± 1	5 ± 3
191	40 ± 3	32 ± 8	3 ± 2
172	38 ± 5	37 ± 10	2 ± 2
162	33 ± 0	28 ± 1	4 ± 1

* All data are the percent of antigen (^{125}I-DNP-BSA) bound by a 1:50 serum dilution. Data are the average of duplicate determinations ± SD.

some of the mice, none of them produced any significant antibody of the low responder (b) allotype.

Discussion

Allophenic mice provide an environment in which histoincompatible cells can coexist in a single animal. Since the cells coexist from a very early stage of development, allophenic mice are potentially chimeric in all their tissues and organs. They are therefore termed primary chimeras and are different from secondary chimeras, such as bone marrow chimeras, which are chimeric in only their lymphomyeloid system (19). The mechanisms for the establishment and maintenance of tolerance in allophenic mice or in bone marrow chimeric mice are unknown. However, it is clear that functional tolerance of the histoincompatible cells does exist in both types of mice.

In the present study, we have shown that the presence of two histoincompatible cell types in allophenic mice, one from a high responder strain, and one from a low responder strain to $DNP_{56}BGG$, is not sufficient to allow the low responder cells to produce anti-DNP antibody. It is not known if the genes controlling the immune response to $DNP_{56}BGG$ are expressed at the level of the T cell, the B cell, the macrophage, or all three. Low responder (C57BL/6) B cells can make anti-DNP antibody when the DNP group is on a carrier other than BGG (20). Thus, the inability to respond is probably at the T-cell or T cell-B cell interaction level. However, as is shown in the present study, in allophenic mice containing high responder T cells capable of recognizing the carrier and low responder B cells capable of recognizing the hapten, antibody is not produced by the low responder B cells. A likely explanation is that the high responder T cells and low responder B cells cannot cooperate across a histocompatibility difference barrier in response to an antigen with genetic control residing in immune response gene(s) in the *H-2* complex. This interpretation would be in full agreement with the recently reported data on the (T,G)-A–L system (10).

Received for publication 7 March 1978.

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INDEX TO AUTHORS

A

Adams, D. D. See Knight and Adams 1653

Aguet, M., Andersson, L. C., Andersson, R., Wight, E., Binz, H., and Wigzell, H. Induction of specific immune unresponsiveness with purified mixed leukocyte culture-activated T lymphoblasts as autoimmunogen. II. An analysis of the effects measured at the cellular and serological levels 50

Albrecht, Paul. See Verbonitz, Ennis, Hicks, and Albrecht 265

Alper, Chester A. See Voelkel, Levine, Alper, and Tashjian 1078

Alspaugh, Margaret A., Jensen, Fred C., Rabin, Harvey, and Tan, Eng M. Lymphocytes transformed by Epstein-Barr virus. Induction of nuclear antigen reactive with antibody in rheumatoid arthritis 1018

Althage, A. See Zinkernagel, Callahan, Althage, Cooper, Klein, and Klein 882

—. See Zinkernagel, Callahan, Althage, Cooper, Streilein, and Klein 897

Andersson, Jan, Coutinho, Antonio, and Melchers, Fritz. The switch from IgM to IgG secretion in single mitogen-stimulated B-cell clones 1744

Andersson, L. C. See Aguet, Andersson, Andersson, Wight, Binz, and Wigzell 50

Andersson, R. See Aguet, Andersson, Andersson, Wight, Binz, and Wigzell 50

André-Schwartz, Janine. See Datta, Manny, Andrzejewski, André-Schwartz, and Schwartz 854

Andrzejewski, Chester. See Datta, Manny, Andrzejewski, André-Schwartz, and Schwartz 854

Arnold, Karen F. See Davis, Ziegler, and Arnold 1007

Asofsky, Richard. See Mathieson, Campbell, Potter, and Asofsky 1267

—. See Sieckmann, Asofsky, Mosier, Zitron, and Paul 814

Assisi, F. See Zan-Bar, Vitetta, Assisi, and Strober 1374

Astle, Clinton M. See Harrison, Astle, and Delaittre 1526

Atkins, Robert C. See Holdsworth, Thomson, Glasgow, Dowling, and Atkins 98

Austen, K. Frank. See Weiler, Yurt, Fearon, and Austen 409

B

Bach, Fritz H. See Zarling and Bach 1334

Bailey, Donald W. See Wettstein, Bailey, Mobraaten, Klein, and Frelinger 1395

Baltimore, David. See Rosenberg and Baltimore 1126

Bamberger, Stephan. See Block, Jaksche, Bamberger, and Ruhenstroth-Bauer 541

Bartholomew, Richard M., Esser, Alfred F., and Müller-Eberhard, Hans J. Lysis of oncornaviruses by human serum. Isolation of the viral complement (C1) receptor and identification as p15E 844

Basch, Ross S., Kadish, Julian L., and Goldstein, Gideon. Hematopoietic thymocyte precursors. IV. Enrichment of the precursors and evidence for heterogeneity 1843

Batey, M. E. See Engleman, McMichael, Batey, and McDevitt 137

Benacerraf, Baruj. See Germain, Thèze, Kapp, and Benacerraf 123

—. See Pierres, Germain, Dorf, and Bennacerraf 656

Bennett, Michael. See Kumar, Goldschmidt, Eastcott, and Bennett 422

Berntson, Tom J. See Warner, Berntson, Eakley, McIvor, and Newton 1849

Berzofsky, Jay A. See Pisetsky, Berzofsky, and Sachs 396

Bieber, C. P. See Slavin, Reitz, Bieber, Kaplan, and Strober 700

Biggin, Judith. See Delovitch, Biggin, and Fung 1198

Binz, Hans, and Wigzell, Hans. Induction of specific immune unresponsiveness with purified mixed leukocyte cultured-activated T lymphoblasts as autoimmunogen. III. Proof for the existence of autoanti-idiotypic killer T cells and transfer of suppression to normal syngeneic recipients by T or B lymphocytes 63

—. See Aguet, Andersson, Andersson, Wight, Binz, and Wigzell 50

—. See Frischknecht, Binz, and Wigzell 500

Birshtein, Barbara K. See Francus, Dharmgrongartama, Campbell, Scharff, and Birshtein 1535

Black, Samuel J., van der Loo, Wessel, Loken, Michael R., and Herzenberg, Leonard A. Expression of IgD by murine lymphocytes. Loss of surface IgD indicates maturation of memory B cells. 984

Blanden, Robert V., and Kees, Ursula. Cytotoxic T-cell responses show more restricted specificity for self than for non-self *H-2D*-coded antigens 1661

Blank, Kenneth J. See Duran-Reynals, Lilly, Bosch, and Blank 459

Block, Lutz H., Jaksche, Herbert, Bamberger, Stephan, and Ruhenstroth-Bauer, Gerhard. Human migration inhibitory factor: purification and immunochemical characterization 541
Bloom, Barry R. See Ju, Udem, Rager-Zisman, and Bloom 1637
Blumencranz, Steven J. See Steinman, Blumencranz, Machtinger, Fried, and Cohn 297
Bockman, R. See Kurland and Bockman 952
Bodel, Phyllis. Spontaneous pyrogen production by mouse histiocytic and myelomonocytic tumor cell lines in vitro 1503
Bosch, Albert. See Duran-Reynals, Lilly, Bosch, and Blank 459
Botzenhardt, U., Klein, J., and Ziff, M. Primary in vitro cell-mediated lympholysis reaction of NZB mice against unmodified targets syngeneic at the major histocompatibility complex 1435
Bouma, Bonno N. See Revak, Cochrane, Bouma, and Griffin 719
Bourguignon, Lilly, Y. W., Hyman, Robert, Trowbridge, Ian, and Singer, S. J. Localization of T25 glycoprotein in wild-type and Thy 1^- mutant cells by immunofluorescence and immunoelectron microscopy 1348
Boyse, Edward A. See Scheid, Goldstein, and Boyse 1727
—. See Tung, O'Donnell, Fleissner, and Boyse 1280
Braciale, T. J., and Yap, K. L. Role of viral infectivity in the induction of influenza virus-specific cytotoxic T cells 1236
Braley-Mullen, Helen. Antigen requirements for induction of B-memory cells. Studies with dinitrophenyl coupled to T-dependent and T-independent carriers 1824
Breard, Jacqueline. See Nathan, Chess, Hillman, Clarke, Breard, Merler, and Housman 324
Bubbers, J. Eric, Chen, Suzie, and Lilly, Frank. Nonrandom inclusion of *H-2K* and *H-2D* antigens in Friend virus particles from mice of various strains 340
Bulterman, Dini. See van der Meer, Bulterman, van Zwet, Elzenga-Claasen, and van Furth 271
Burakoff, Steven. See Mescher, Sherman, Lemonnier, and Burakoff 946

C

Calkins, C. E., and Stutman, O. Changes in suppressor mechanisms during postnatal development in mice 87
Callahan, G. N. See Zinkernagel, Callahan, Althage, Cooper, Klein, and Klein 882
—. See Zinkernagel, Callahan, Althage, Cooper, Streilein, and Klein 897
Campbell, P. S. See Mathieson, Campbell, Potter, and Asofsky 1267
Campbell, Richard. See Francus, Dharmgrongartama, Campbell, Scharff, and Birshtein 1535
Cancro, Michael P., Gerhard, Walter, and Klinman, Norman R. The diversity of the influenza-specific primary B-cell repertoire in BALB/c mice 776
—. Sigal, Nolan H., and Klinman, Norman R. Differential expression of an equivalent clonotype among BALB/c and C57BL/6 mice 1
Cantor, H., McVay-Boudreau, L., Hugenberger, J., Naidorf, K., Shen, F. W., and Gershon, R. K. Immunoregulatory circuits among T-cell sets. II. Physiologic role of feedback inhibition in vivo: absence in NZB mice 1116
—. See Eardley, Hugenberger, McVay-Boudreau, Shen, Gershon, and Cantor 1106
Carlo, Jaime R. See Conrad, Carlo, and Ruddy 1792
Chandler, P. R. See Hurme, Hetherington, Chandler, and Simpson 758
—. See Hurme, Chandler, Hetherington, and Simpson 768
Chang, K-P. and Dwyer, D. M. *Leishmania donovani*. Hamster macrophage interactions in vitro: cell entry, intracellular survival, and multiplication of amastigotes 515
Chase, Catharine M. See Russell, Chase, Colvin, and Plate 1449, 1469
Chen, Suzie. See Bubbers, Chen, and Lilly 340
Chess, Leonard. See Evans, Chess, Levine, and Schlossman 605
—. See Nathan, Chess, Hillman, Clarke, Breard, Merler, and Housman 324
Claman, Henry N. See Miller, Sy, and Claman 788
Clarke, Bryan. See Nathan, Chess, Hillman, Clarke, Breard, Merler, and Housman 324
Cochrane, Charles G. See Lennon, Seybold, Linstrom, Cochrane, and Ulevitch 973
—. See Revak, Cochrane, Bouma, and Griffin 719
Cohn, Zanvil A. See Gordon and Cohn 1175
—. See Steinman, Blumencranz, Machtinger, Fried, and Cohn 297
—. See Steinman, Machtinger, Fried, and Cohn 279
Colvin, Robert B. See Russell, Chase, Colvin, and Plate 1449, 1469
Conliffe, McGregor A. See Gipson, Imamura, Conliffe, and Martin 1363
Conrad, Daniel H., Carlo, Jaime R., and Ruddy, Shaun. Interaction of β1H globulin with cell-bound C3b. Quantitative analysis of binding and influence of alternative path-

way components on binding 1792

Coons, Albert H. See Shek and Coons 1213

—. See Shek, Waltenbaugh, and Coons 1228

Cooper, Max D. See Grossi Webb, Zicca, Lydyard, Moretta, Mingari, and Cooper 1405

Cooper, Neil R. See Ziccardi and Cooper 385

Cooper, S. See Zinkernagel, Callahan, Althage, Cooper, Klein, and Klein 882

—. See Zinkernagel, Callahan, Althage, Cooper, Streilein, and Klein 897

Cotran, Ramzi S. See Schreiner, Cotran, Pardo, and Unanue 369

Coutinho, Antonio. See Andersson, Coutinho, and Melchers 1774

Cowing, Carol. See Singer, Cowing, Hathcock, Dickler, and Hodes 1611

Crago, Sylvia S., Kulhavy, Rose, Prince, Shirley J., and Mestecky, Jiri. Secretory component on epithelial cells is a surface receptor for polymeric immunoglobulins 1832

Cramer, M. See Krawinkel, Cramer, Melchers, Imanishi-Kari, and Rajewsky 1341

D

Daniele, Ronald P., Holian, Sandra K., and Nowell, Peter C. A potassium ionophore (nigericin) inhibits stimulation of human lymphocytes by mitogens 571

Danø, Keld, and Reich, E. Serine enzymes released by cultured neoplastic cells 745

Datta, Syamal K., Manny, Noga, Andrzejewski, Chester, André-Schwartz, Janine, and Schwartz, Robert S. Genetic studies of autoimmunity and retrovirus expression in crosses of New Zealand black mice. I. Xenotropic virus 854

—. McConahey, Patricia J., Manny, Noga, Theofilopoulos, Argyrios, N., Dixon, Frank J., and Schwartz, Robert S. Genetic studies of autoimmunity and retrovirus expression in crosses of New Zealand black mice. II. The viral envelope glycoprotein gp70 872

David, Chella S. See Okuda and David 1028

Davis, Charles E., Ziegler, Elizabeth J., and Arnold, Karen F. Neutralization of meningococcal endotoxin by antibody to core glycolipid 1007

Deak, Beverly D., Meruelo, Daniel, and McDevitt, Hugh O. Expression of a single major histocompatibility complex locus controls the immune response to poly-L-(tyrosine, glutamic acid)-poly-DL-alanine—poly-L-lysine 599

Dee, Robert R. See Trinchieri, Santoli, Dee, and Knowles 1299

Delaittre, Joan A. See Harrison, Astle, and Delaittre 1526

Delovitch, Terry L., Biggin, Judith, and Fung, Foon-Yee. In vitro analysis of allogeneic lymphocyte interaction. II. *I*-region control of the activity of a B-cell-derived *H-2*-restricted allogeneic effect factor and its receptor during B-cell activation 1198

Deuel, Esther. See Lindenmann, Deuel, Fanconi, and Haller 531

Dharmgrongartama, B. See Francus, Dharmgrongartama, Campbell, Scharff, and Birshtein 1535

Dickler, Howard B. See Singer, Cowing, Hathcock, Dickler, and Hodes 1611

Dixon, Frank J. See Datta, McConahey, Manny, Theofilopoulos, Dixon, and Schwartz 872

—. See Eisenberg, Tan, and Dixon 582

Dorf, Martin E. See Pierres, Germain, Dorf, and Benacerraf 656

Dorie, Mary J. See Maloney, Dorie, Lamela, Rogers, and Patt 1189

Dowling, John P. See Holdsworth, Thomson, Glasgow, Dowling, and Atkins 98

Duran-Reynals, Maria L., Lilly, Frank, Bosch, Albert, and Blank, Kenneth J. The genetic basis of susceptibility to leukemia induction in mice by 3-methylcholanthrene applied percutaneously 459

Dwyer, D. M. See Chang and Dwyer 515

E

Eakley, Lois. See Warner, Berntson, Eakley, McIvor, and Newton 1849

Eardley, D. D., Hugenberger, J., McVay-Boudreau, L., Shen, F. W., Gershon, R. K., and Cantor, H. Immunoregulatory circuits among T-cell sets. I. T-helper cells induce other T-cell sets to exert feedback inhibition 1106

Eastcott, Jean W. See Kumar, Goldschmidt, Eastcott, and Bennett 422

Edelson, Paul J., and Erbs, Cheryl. Plasma membrane localization and metabolism of alkaline phosphodiesterase I in mouse peritoneal macrophages 77

Eisenberg, Robert A., Tan, Eng M., and Dixon, Frank J. Presence of anti-Sm reactivity in autoimmune mouse strains 582

Elfenbein, G. J. See Volf, Sensenbrenner, Sharkis, Elfenbein, and Scher 940

Elzenga-Claasen, Ine. See van der Meer, Bulterman, van Zwet, Elzenga-Claasen, and van Furth 271

Emeson, Eugene E. Migratory behavior of lymphocytes with specific reactivity to alloantigens. II. Selective recruitment to lymphoid cell allografts and their draining lymph nodes 13

Emlen, Woodruff, and Mannik, Mart. Kinetics and mechanisms for removal of circulating single-stranded DNA in mice 684

Engleman, Edgar G., McMichael, A. J., Battey, M. E., and McDevitt, H. O. A suppressor T cell of the mixed lymphocyte reaction in man specific for the stimulating alloantigen. Evidence that identity at HLA-D between suppressor and responder is required for suppression 137

—. McMichael, Andrew J., and McDevitt, Hugh O. Suppression of the mixed lymphocyte reaction in man by a soluble T-cell factor. Specificity of the factor for both responder and stimulator 1037

Engvall, Eva, Ruoslahti, Erkki, and Miller, Edward J. Affinity of fibronectin to collagens of different genetic types and to fibrinogen 1584

Ennis, Francis A. See Verbonitz, Ennis, Hicks, and Albrecht 265

Erbs, Cheryl. See Edelson, and Erbs 77

Esser, Alfred F. See Bartholomew, Esser, and Müller-Eberhard 844

Evans, Robert L., Chess, Leonard, Levine, Herbert, and Schlossman, Stuart F. Antibody-dependent cellular cytotoxicity by allosensitized human T cells 605

F

Fanconi, Sergio. See Lindenmann, Deuel, Fanconi, and Haller 531

Fearon, Douglas T. See Weiler, Yurt, Fearon, and Austen 409

Feldmann, Marc. See Kontiainen, and Feldmann 110

Fernandez, Carmen, and Möller, Göran. Immunological unrespnsiveness to native dextran B512 in young animals of dextran high responder strains is due to lack of Ig receptor expression. Evidence for a nonrandom expression of V-genes 645

Fischetti, Vincent A. Streptococcal M protein extracted by nonionic detergent. III. Correlation between immunological cross-reactions and structural similarities with implications for anti-phagocytosis 1771

Fleissner, Erwin. See Tung, O'Donnell, Fleissner, and Boyse 1280

Foley, Rosemary. See Werb, Foley, and Munck 1684

Francus, Tova, Dharmgrongartama, B., Campbell, Richard, Scharff, Matthew D., and Birshtein, Barbara K. IgG_{2a}-producing variants of an IgG_{2b}-producing mouse myeloma cell line 1535

Frasch, Carl E., and Robbins, Joan D. Protection against group B meningococcal disease. II. Infection and resulting immunity in a guinea pig model 619

—. Protection against group B meningococcal disease. III. Immunogenicity of serotype 2 vaccines and specificity of protection in a guinea pig model 629

Frelinger, Jeffrey A. See Wettstein, Bailey, Mobraaten, Klein, and Frelinger 1395

Fried, Jerrold. See Steinman, Blumencranz, Machtinger, Fried, and Cohn 297

—. See Steinman, Machtinger, Fried, and Cohn 279

Frischknecht, Hannes, Binz, Hans, and Wigzell, Hans. Induction of specific transplantation immune reactions using anti-idiotypic antibodies 500

Fuks, Z. See Slavin, Fuks, Kaplan, and Strober. 963

Fung, Foon-Yee. See Delovitch, Biggin, and Fung 1198

G

Gardner, Donald E. See Hatch, Gardner, and Menzel 182

Gerhard, Walter. See Cancro, Gerhard, and Klinman 776

Germain, Ronald N., Thèze, Jacques, Kapp, Judith A., and Benacerraf, Baruj. Antigen-specific T-cell-mediated suppression. I. Induction of L-glutamic $acid^{60}$-L-$alanine^{30}$-L-$tyrosine^{10}$ specific suppressor T cells in vitro requires both antigen-specific T-cell-suppressor factor and antigen 123

—. See Pierres, Germain, Dorf, and Benacerraf 656

Gershon, R. K. See Cantor, McVay-Boudreau, Hugenberger, Naidorf, Shen, and Gershon 1116

—. See Eardley, Hugenberger, McVay-Boudreau, Shen, Gershon, and Cantor 1106

Ginsberg, M. H., and Henson, P. M. Enhancement of platelet response to immune complexes and IgG aggregates by lipid A-rich bacterial lipopolysaccharides 207

Gipson, Thomas G., Imamura, Masahiro, Conliffe, McGregor A., and Martin, W. John. Lung tumor-associated derepressed alloantigen coded for by the *K* region of the *H-2* major histocompatibility complex 1363

Glasgow, Eric F. See Holdsworth, Thomson, Glasgow, Dowling, and Atkins 98

Goldschmidt, Leonard. See Kumar, Goldschmidt, Eastcott, and Bennett 422

Goldstein, Allan L. See Pazmiño, Ihle, and Goldstein 708

Goldstein, Gideon. See Scheid, Goldstein, and Boyse 1727

—. See Basch, Kadish, and Goldstein 1843

Golstein, Pierre. See MacLennan and Golstein 1551

—. See Wagner, Starzinski-Powitz, Röllinghoff, Golstein, and Jakob 251

Goodman, J. R. See Roubinian, Talal, Greenspan, Goodman, and Siiteri 1568

Goodman, Michael G., Parks, D. Elliot, and Weigle, William O. Immunologic responsiveness of the C3H/HeJ mouse: differential ability of butanol-extracted lipopolysaccharide (LPS) to evoke LPS-mediated effects 800

Gordon, Saimon, and Cohn, Zanvil A. Bacille Calmette-Guérin infection in the mouse. Regulation of macrophage plasminogen activator by T lymphocytes and specific antigen 1175

Granelli-Piperno, Angela. See Vassalli, Granelli-Piperno, Griscelli, and Reich 1285

Granick, S. See Grieninger and Granick 1806

Greenspan, J. S. See Roubinian, Talal, Greenspan, Goodman, and Siiteri 1568

Grieninger, Gerd, and Granick, S. Synthesis and secretion of plasma proteins by embryonic chick hepatocytes: changing patterns during the first three days of culture 1806

Griffin, John H. See Revak, Cochrane, Bouma, and Griffin 719

Griscelli, Claude. See Vassalli, Granelli-Piperno, Griscelli, and Reich 1285

Grossi, Carlo E., Webb, Susan R., Zicca, Antonio, Lydyard, Peter M., Moretta, Lorenzo, Mingari, M. Cristina, and Cooper, Max D. Morphological and histochemical analyses of two human T-cell subpopulations bearing receptors for IgM or IgG 1405

H

Haas, Werner. See von Boehmer, Haas, and Pohlit 1291

Hall, Joe. see Orlans, Peppard, Reynolds, and Hall 588

Haller, Otto. See Lindenmann, Deuel, Fanconi, and Haller 531

Hamburg, S. I., Manejias, R. E., and Rabinovitch, M. Macrophage activation: increased ingestion of IgG-coated erythrocytes after administration of interferon inducers to mice 593

Harrison, David E., Astle, Clinton M., and Delaittre, Joan A. Loss of proliferative capacity in immunohemopoietic stem cells caused by serial transplantation rather than aging 1526

Hatch, Gary E., Gardner, Donald E., and Menzel, Daniel B. Chemiluminescence of phagocytic cells caused by *N*-formylmethionyl peptides 182

Hathcock, Karen S. See Singer, Cowing, Hathcock, Dickler, and Hodes 1611

Henkart, Pierre A. See Schmitt-Verhulst, Pettinelli, Henkart, Lunney, and Shearer 352

Henning, Margaret M. See McKenzie and Henning 611

Henson, P. M. See Ginsberg and Henson 207

Herzenberg, Leonard A. See Black, van der Loo, Loken, and Herzenberg 984

Hetherington, C. M. See Hurme, Hetherington, Chandler, and Simpson 758

Hetherington, C. M. See Hurme, Chandler, Hetherington, and Simpson 768

Hicks, John T. See Verbonitz, Ennis, Hicks, and Albrecht 265

Hillman, Diane G. See Nathan, Chess, Hillman, Clarke, Breard, Merler, and Housman 324

Hodes, Richard J. See Singer, Cowing, Hathcock, Dickler, and Hodes 1611

Holdsworth, Stephen R., Thomson, Napier M., Glasgow, Eric F., Dowling, John P., and Atkins, Robert C. Tissue culture of isolated glomeruli in experimental crescentic glomerulonephritis 98

Holian, Sandra K. See Daniele, Holian, and Nowell 571

Housman, David E. See Nathan, Chess, Hillman, Clarke, Breard, Merler, and Housman 324

Hugenberger, J. See Cantor, McVay-Boudreau, Hugenberger, Naidorf, Shen, and Gershon 1116

—. See Eardley, Hugenberger, McVay-Boudreau, Shen, Gershon, and Cantor 1106

Hurme, M., Hetherington, C. M., Chandler, P. R., and Simpson, Elizabeth. Cytotoxic T-cell responses to H-Y: mapping of the Ir genes 758

—. Chandler, P. R., Hetherington, C. M., and Simpson, Elizabeth. Cytotoxic T-cell responses to H-Y: correlation with the rejection of syngeneic male skin grafts 768

Hyman, Robert. See Bourguignon, Hyman, Trowbridge, and Singer 1348

I

Imamura, Masahiro. See Gipson, Imamura, Conliffe, and Martin 1363

Imanishi-Kari, T. See Krawinkel, Cramer, Melchers, Imanishi-Kari, and Rajewsky 1341

Ihle, James N. See Pazmiño, Ihle, and Goldstein 708

Inman, J. K. See Merchant, Snippe, Lizzio, and Inman 1755

Iscove, N. N., and Melchers, F. Complete replacement of serum by albumin, tranferrin, and soybean lipid in cultures of lipopolysaccharide-reactive B lymphocytes 923

J

Jackson, Graham Douglas Fisher. See Lemaître-Coelho, Jackson, and Vaerman 934

Jaffe, Eric A., and Mosher, Deane F. Synthesis of fibroncetin by cultured human endothelial cells 1779

Jakob, Hedwig. See Wagner, Starzinski-Powitz, Röllinghoff, Golstein, and Jakob 251

Jaksche, Herbert. See Block, Jaksche, Bamberger, and Ruhenstroth-Bauer 541

Janeway, Charles A., Jr., Murphy, Patricia D., Kemp, John, and Wigzell, Hans. T cells specific for hapten-modified self are precommitted for self major histocompatibility complex antigens before encounter with the hapten 1065

Jarowski, Charles I. See Ross, Jarowski, Rabellino, and Winchester 730

Jegasothy, Brian V. See Wagshal, Jegasothy, and Waksman 171

Jensen, Fred C. See Alspaugh, Jensen, Rabin, and Tan 1018

Jondal, Mikael, and Targan, Stephan. In vitro induction of cytotoxic effector cells with spontaneous killer cell specificity 1621

Jones, Patricia P. See Meruelo, Nimelstein, Jones, Lieberman, and McDevitt 470

Jones, Thomas C. See Masur and Jones 157

Ju, Grace, Udem, Stephen, Rager-Zisman, Bracha, and Bloom, Barry R. Isolation of a heterogeneous population of temperature-sensitive mutants of measles virus from persistently infected human lymphoblastoid cell lines 1637

K

Kabat, Elvin A. See Kisailus and Kabat 830

Kadish, Julian L. See Basch, Kadish, and Goldstein 1843

Kaplan, Allen P. See Thompson, Mandle, and Kaplan 488

Kaplan, H. S. See Slavin, Fuks, Kaplan, and Strober 963

—. See Slavin, Reitz, Bieber, Kaplan, and Strober 700

Kapp, Judith A. Immunosuppressive factors from lymphoid cells of nonresponder mice primed with L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}. IV. Lack of strain restrictions among allogeneic, nonresponder donors and recipients 997

—. See Germain, Thèze, Kapp, and Benacerraf 123

Kappler, John W. See Marrack and Kappler 1596

—. See Swierkosz, Rock, Marrack, and Kappler 554

Kees, Ursula. See Blanden and Kees 1661

Kemp, John. See Janeway, Murphy, Kemp, and Wigzell 1065

Kimura, Arthur K., and Wigzell, Hans. Cell surface glycoproteins of murine cytotoxic T lymphocytes. I. T 145, a new cell surface glycoprotein selectively expressed on Ly $1^{-}2^{+}$ cytotoxic T lymphocytes 1418

Kisailus, Edward C., and Kabat, Elvin A. Immunochemical studies on blood groups. LXVI. Competitive binding assays of A_1 and A_2 blood group substances with insolubilized anti-A serum and insolubilized A agglutinin from *Dolichos biflorus* 830

Klassen, L. W. See Ravechè, Steinberg, Klassen, and Tjio 1487

Klein, Jan. See Botzenhardt, Klein, and Ziff 1435

—. See Wettstein, Bailey, Mobraaten, Klein, and Frelinger 1395

—. See Zinkernagel, Callahan, Althage, Cooper, Klein, and Klein 882

—. See Zinkernagel, Callahan, Althage, Cooper, Streilein, and Klein 897

Klein, P. A. See Zinkernagel, Callahan, Althage, Cooper, Klein, and Klein 882

Klinman, Norman R. See Cancro, Gerhard, and Klinman 776

—. See Cancro, Sigal, and Klinman 1

Knight, J. G., and Adams, D. D. Three genes for lupus nephritis in NZB × NZW mice 1653

Knowles, Barbara B. See Trinchieri, Santoli, Dee, and Knowles 1299

Kontiainen, Sirkka, and Feldmann, Marc. Suppressor-cell induction in vitro. IV. Target of antigen-specific suppressor factor and its genetic relationships 110

Krammer, Peter H. Alloantigen receptors on activated T cells in mice. I. Binding of alloantigens and anti-idiotypic antibodies to the same receptors 25

Krawinkel, U., Cramer, M., Melchers, I., Imanishi-Kari, T., and Rajewsky, K. Isolated hapten-binding receptors of sensitized lymphocytes. III. Evidence for idiotypic restriction of T-cell receptors 1341

Kulhavy, Rose. See Czago, Kulhavy, Prince and Mestecky 1832

Kumar, Vinay, Goldschmidt, Leonard, Eastcott, Jean W., and Bennett, Michael. Mechanisms of genetic resistance to Friend virus leukemia in mice. IV. Identification of a gene (*Fv-3*) regulating immunosuppression in vitro, and its distinction from *Fv-2* and genes regulating marrow allograft reactivity 422

Kurland, J. I. and Bockman, R. Prostaglandin E production by human blood monocytes and mouse peritoneal macrophages 952

L

Lamela, Rosito A. See Maloney, Dorie, Lamela, Rogers, and Patt 1189

Largen, Michael. See Watson, Largen, and McAdam 39

Lee, S. K., and Oliver, R. T. D. Autologous leukemia-specific T-cell-mediated lymphocytotoxicity in patients with acute myelogonous leukemia 912

Lemaître-Coelho, Isabel, Jackson, Graham Douglas Fisher, and Vaerman, Jean-Pierre. High levels of secretory IgA and free secretory component in the serum of rats with bile duct obstruction 934

Lemonnier, Francois. See Mescher, Sherman, Lemonnier, and Burakoff 946

Lennon, Vanda A., Seybold, Marjorie E., Lindstrom, Jon M., Cochrane, Charles, and Ulevitch, Richard. Role of complement in the pathogenesis of experimental autoimmune myasthenia gravis 973

Levine, Herbert. See Evans, Chess, Levine, and Schlossman 605

Levine, Lawrence. See Voelkel, Levine, Alper, and Tashjian 1078

Lieberman, Miriam. See Meruelo, Nimelstein, Jones, Lieberman, and McDevitt 470

Lilly, Frank. See Bubbers, Chen, and Lilly 340

—. See Duran-Reynals, Lilly, Bosch, and Blank 459

Lindenmann, Jean, Deuel, Esther, Fanconi, Sergio, and Haller, Otto. Inborn resistance of mice to myxoviruses: macrophages express phenotype in vitro 531

Lindstrom, Jon M. See Lennon, Seybold, Lindstrom, Cochrane, and Ulevitch 973

Lizzio, E. F. See Merchant, Snippe, Lizzio, and Inman 1755

LoBuglio, Albert F. See Weiss, Rustagi, and LoBuglio 316

Loken, Michael R. See Black, van der Loo, Loken, and Herzenberg 984

Lunney, Joan K. See Schmitt-Verhulst, Pettinelli, Henkart, Lunney, and Shearer 352

Lydyard, Peter M. See Grossi, Webb, Zicca, Lydyard, Moretta, Mingari, and Cooper 1405

M

Machtinger, Beverly G. See Steinman, Blumencranz, Machtinger, Fried, and Cohn 297

—. See Steinman, Machtinger, Fried, and Cohn 279

MacLennan, Ian C. M., and Golstein, Pierre. Requirement for hexose, unrelated to energy provision, in T-cell-mediated cytolysis at the lethal hit stage 1551

Maloney, Mary A., Dorie, Mary J., Lamela, Rosito A., Rogers, Zora R., and Patt, Harvey M. Hematopoietic stem cell regulatory volumes as revealed in studies of the bg^J/bg^J: W/W^v chimera 1189

Mandle, Robert, Jr. See Thompson, Mandle, and Kaplan 488

Manejias, R. E. See Hamburg, Manejias, and Rabinovitch 593

Mannik, Mart. See Emlen and Mannik 684

Manny, Noga. See Datta, Manny, Andrzejewski, André-Schwartz, and Schwartz 854

—. See Datta, McConahey, Manny, Theofilopoulos, Dixon, and Schwartz 872

Marrack, Philippa, and Kappler, John W. The role of H-2-linked genes in helper T-cell function. III. Expression of immune response genes for trinitrophenyl conjugates of poly-L(Tyr, Glu)-poly-D,L-Ala--poly-L-Lys in B cells and macrophages 1596

—. See Swierkosz, Rock, Marrack, and Kappler 554

Martin, W. John. See Gipson, Imamura, Conliffe, and Martin 1363

Masur, Henry, and Jones, Thomas C. The interaction in vitro of *Pneumocystis carinii* with macrophages and L cells 157

Mathieson, B. J., Campbell, P. S., Potter, M., and Asofsky, R. Expression of Ly 1, Ly 2, Thy 1, and TL differentiation antigens on mouse T-cell tumors 1267

McAdam, Keith P. W. J. See Watson, Largen, and McAdam 39

McConahey, Patricia J. See Datta, McConahey, Manny, Theofilopoulos, Dixon, and Schwartz 872

McDevitt, Hugh O. See Deak, Meruelo, and McDevitt 599

—. See Engleman, McMichael, Batey, and McDevitt 137

—. See Engleman, McMichael, and McDevitt 1037

—. See Meruelo, Nimelstein, Jones, Lieberman, and McDevitt 470

McIvor, Judith L. See Warner, Berntson, Eakley, McIvor, and Newton 1849

McKenzie, Ian F. C., and Henning, Margaret M. The differential destructive and enhancing effects of anti-H-2K, H-2D, and anti-Ia antisera on murine skin allografts 611

McLaren, Diane J. See Ramalho-Pinto, McLaren, and Smithers 147

McMichael, Andrew J. See Engleman, McMichael, Batey, and McDevitt 137

—. See Engleman, McMichael, and McDevitt 1037

McVay-Boudreau, L. See Cantor, McVay-Boudreau, Hugenberger, Naidorf, Shen, and Gershon 1116

—. See Eardley, Hugenberger, McVay-Boudreau, Shen, Gershon, and Cantor 1106

Melchers, Fritz. See Andersson, Coutinho, and Melchers 1744

—. See Iscove and Melchers 923

Melchers, I. See Krawinkel, Cramer, Melchers, Imanishi-Kari, and Rajewsky 1341

Menzel, Daniel B. See Hatch, Gardner, and Menzel 182

Merchant, B., Snippe, H., Lizzio, E. F., and Inman, J. K. Cellular and molecular requirements for X-linked, hapten-specific B-cell blockade in CBA/N mice 1755

Merler, Ezio. See Nathan, Chess, Hillman, Clarke, Breard, Merler, and Housman 324

Meruelo, Daniel, Nimelstein, Stephen H., Jones, Patricia P., Lieberman, Miriam, and McDevitt, Hugh O. Increased synthesis and expression of H-2 antigens on thymocytes as a result of radiation leukemia virus infection: a possible mechanism for *H-2*-linked control of virus-induced neoplasia 470

—. See Deak, Meruelo, and McDevitt 599

Mescher, Matthew, Sherman, Linda, Lemonnier, Francois, and Burakoff, Steven. The induction of secondary cytolytic T lymphocytes by solubilized membrane proteins 946

Mestecky, Jiri. See Crago, Kulhavy, Prince, and Mestecky 1832

Metcalf, Donald. See Rabellino, Ross, Trang, Williams, and Metcalf 434

Miller, Edward J. See Engvall, Ruoslahti, and Miller 1584

Miller, Stephen D., Sy, Man-Sun, and Claman, Henry N. Genetic restrictions for the induction of suppressor T cells by hapten-modified lymphoid cells in tolerance to 1-fluoro-2,4-dinitrobenzene contact sensitivity. Role of the *H-2D* region of the major histocompatibility complex 788

Mingari, M. Cristina. See Grossi, Webb, Zicca, Lydyard, Moretta, Mingari, and Cooper 1405

Mobraaten, Larry E. See Wettstein, Bailey, Mobraaten, Klein, and Frelinger 1395

Möller, Göran. See Fernandez and Möller 645

Moretta, Lorenzo. See Grossi, Webb, Zicca, Lydyard, Moretta, Mingari, and Cooper 1405

Mosher, Deane F. See Jaffe and Mosher 1779

Mosier, Donald E. See Sieckmann, Asofsky, Mosier, Zitron, and Paul 814

Müller-Eberhard, Hans J. See Bartholomew, Esser, and Müller-Eberhard 844

Munck, Allan. See Werb, Foley, and Munck. 1684

Murgita, Robert A. See Peck, Murgita, and Wigzell 667

Murphy, Patricia D. See Janeway, Murphy, Kemp, and Wigzell 1065

N

Nachman, Ralph. See Polley and Nachman 1713

Naidorf, K. See Cantor, McVay-Boudreau, Hugenberger, Naidorf, Shen, and Gershon 1116

Narayanan, P. R., and Sundharadas, G. Differential effects of polyadenylic: polyuridylic acid and lipopolysaccharide on the generation of cytotoxic T lymphocytes 1355

Nathan, David G., Chess, Leonard, Hillman, Diane G., Clarke, Bryan, Breard, Jacqueline, Merler, Ezio, and Housman, David E. Human erythroid burst-forming unit: T-cell requirement for proliferation in vitro 324

Newton, Robert C. See Warner, Berntson, Eakley, McIvor, and Newton 1849

Nimelstein, Stephen H. See Meruelo, Nimelstein, Jones, Lieberman, and McDevitt 470

Nonaka, Makoto. See Tada, Takemori, Okumura, Nonaka, and Tokuhisa 446

Nowell, Peter C. See Daniele, Holian, and Nowell 571

O

Obata, Yuichi, Stockert, Elisabeth, O'Donnell, Paul V., Okubo, Shuji, Snyder, Harry W., Jr., and Old, Lloyd J. $G_{(\mathrm{RADA1})}$: a new cell surface antigen of mouse leukemia defined by naturally occurring antibody and its relationship to murine leukemia virus 1089

O'Brien, Stephen J., and Simonson, Janice M. *Bvr-1*, a restriction locus of a type C RNA virus in the feline cellular genome: pleiotropic restriction of endogenous BALB virus in cat × mouse somatic cell hybrids 219

O'Donnell, Paul V. See Obata, Stockert, O'Donnell, Okubo, Snyder, and Old 1089

—. See Tung, O'Donnell, Fleissner, and Boyse 1280

Okubo, Shuji. See Obata, Stockert, O'Donnell, Okubo, Snyder, and Old 1089

Okuda, Kenji, and David, Chella S. A new lymphocyte-activating determinant locus expressed on T cells, and mapping in *I-C* subregion 1028

Okumura, Ko. See Tada, Takemori, Okumura, Nonaka, and Tokuhisa 446

Old, Lloyd J. See Obata, Stockert, O'Donnell, Okubo, Snyder, and Old 1089

Oliver, R. T. D. See Lee and Oliver 912

Orlans, Eva, Peppard, Jane, Reynolds, John, and Hall, Joe. Rapid active transport of immunoglobulin A from blood to bile 588

P

Pardo, Victoriano. See Schreiner, Cotran, Pardo, and Unanue 369

Parks, D. Elliot. See Goodman, Parks, and Weigle 800

Patt, Harvey M. See Maloney, Dorie, Lamela, Rogers, and Patt 1189

Paul, William E. See Sieckmann, Asofsky, Mosier, Zitron, and Paul 814

Pazmiño, Nelson H., Ihle, James N., and Goldstein, Allan L. Induction in vivo and in vitro of terminal deoxynucleotidyl transferase by thymosin in bone marrow cells from athymic mice 708

Peck, Ammon B., Murgita, Robert A., and Wigzell, Hans. Cellular and genetic restrictions in the immunoregulatory activity of alpha-fetoprotein. I. Selective inhibition of anti-Ia-associated proliferative reactions 667

Peppard, Jane. See Orlans, Peppard, Reynolds, and Hall 588

Pettinelli, Carla B. See Schmitt-Verhulst, Pettinelli, Henkart, Lunney, and Shearer 352

Pierres, Michel, Germain, Ronald N., Dorf, Martin E., and Benacerraf, Baruj. In vivo effects of anti-Ia alloantisera. I. Elimination of specific suppression by in vivo administration of antisera specific for I-J controlled determinants 656

Pisetsky, David S., Berzofsky, Jay A., and Sachs, David H. Genetic control of the immune response to staphylococcal nuclease. VII. Role of non-*H-2*-linked genes in the control of the anti-nuclease antibody response 396

—, and Sachs, David H. Genetic control of the immune response to staphylococcal nuclease. VIII. Mapping of genes for antibodies to different antigenic regions of nuclease 1517

Plate, Janet M. D. See Russell, Chase, Colvin, and Plate 1449, 1469

Pohlit, Helmut. See von Boehmer, Haas, and Pohlit 1291

Polley, Margaret J., and Nachman, Ralph. The human complement system in thrombin-mediated platelet function 1713

Potter, M. See Mathieson, Campbell, Potter, and Asofsky 1267

Prince, Shirley J. See Crago, Kulhavy, Prince, and Mestecky 1832

R

Rabellino, Enrique M., Ross, Gordon D., Trang, Hoang T. K., Williams, Neil, and Metcalf, Donald. Membrane receptors of mouse leukocytes. II. Sequential expression of membrane receptors and phagocytic capacity during leukocyte differentiation 434

—. See Ross, Jarowski, Rabellino, and Winchester 730

Rabin, Harvey. See Alspaugh, Jensen, Rabin, and Tan 1018

Rabinovitch, M. See Hamburg, Manejias, and Rabinovitch 593

Rager-Zisman, Bracha. See Ju, Udem, Rager-Zisman, and Bloom 1637

Rajewsky, K. See Krawinkel, Cramer, Melchers, Imanishi-Kari, and Rajewsky 1341

Ramalho-Pinto, F. J., McLaren, Diane J., and Smithers, S. R. Complement-mediated killing of schistosomula of *Schistosoma mansoni* by rat eosinophils in vitro 147

Ravché, E. S., Steinberg, A. D., Klassen, L. W., and Tjio, J. H. Genetic studies in NZB mice. I. Spontaneous autoantibody production 1487

Reich, E. See Danø and Reich 745

—. See Vassalli, Granelli-Piperno, Griscelli, and Reich 1285

Reitz, B. See Slavin, Reitz, Bieber, Kaplan, and Strober 700

Revak, Susan D., Cochrane, Charles G., Bouma, Bonno N., and Griffin, John H. Surface and fluid phase activities of two forms of activated Hageman factor produced during contact activation of plasma 719

Reynolds, John. See Orlans, Peppard, Reynolds, and Hall 588

Rich, Robert R. See Seldin and Rich 1671

Robbins, Joan D. See Frasch and Robbins 619, 629

Rock, Kenneth. See Swierkosz, Rock, Marrack, and Kappler 554

Rogers, Zora R. See Maloney, Dorie, Lamela, Rogers, and Patt 1189

Röllinghoff, Martin. See Wagner, Starzinski-Powitz, Röllinghoff, Golstein, and Jakob 251

Rosenberg, Naomi, and Baltimore, David. The effect of helper virus on Abelson virus-induced transformation of lymphoid cells 1126

Ross, Gordon D., Jarowski, Charles I., Rabellino, Enrique M., and Winchester, Robert J. The sequential appearance of Ia-like antigens and two different complement receptors during the maturation of human neutrophils 730

—. See Rabellino, Ross, Trang, Williams, and Metcalf 434

Roubinian, J. R., Talal, N., Greenspan, J. S., Goodman, J. R., and Siiteri, P. K. Effect of castration and sex hormone treatment on survival, anti-nucleic acid antibodies, and glomerulonephritis in NZB/NZW F_1 mice 1568

Ruddy, Shaun. See Conrad, Carlo, and Ruddy 1792

Ruhenstroth-Bauer, Gerhard. See Block, Jaksche, Bamberger, and Ruhenstroth-Bauer 541

Ruoslahti, Erkki. See Engvall, Ruoslahti, and Miller 1584

Russell, Paul S., Chase, Catharine M., Colvin, Robert B., and Plate, Janet M. D. Kidney transplants in mice. An analysis of the immune status of mice bearing long-term, H-2 incompatible transplants 1449

—, Chase, Catharine M., Colvin, Robert B., and Plate, Janet M. D. Induced immune destruction of long-surviving, H-2 incompatible kidney transplants in mice 1469

Rustagi, Pradip K. See Weiss, Rustagi, and LoBuglio 316

S

Sachs, David H. See Pisetsky, Berzofsky, and Sachs 396
—. See Pisetsky and Sachs 1517
Santoli, Daniela. See Trinchieri, Santoli, Dee, and Knowles 1299
—. See Trinchieri and Santoli 1314
Scharff, Matthew D. See Francus, Dharmgrongartama, Campbell, Scharff, and Birshtein 1535
Scheid, Margrit P., Goldstein, Gideon, and Boyse, Edward A. The generation and regulation of lymphocyte populations. Evidence from differentiative induction systems in vitro 1727
Scher, Charles D. Effect of pseudotype on Abelson virus and Kirsten sarcoma virus-induced leukemia 1044
Scher, I. See Volf, Sensenbrenner, Sharkis, Elfenbein, and Scher 940
Schlesinger, Sondra. See van der Zeijst, Stewart, and Schlesinger 1253
Schlossman, Stuart F. See Evans, Chess, Levine, and Schlossman 605
Schmitt-Verhulst, Anne-Marie, Pettinelli, Carla B., Henkart, Pierre A., Lunney, Joan K., and Shearer, Gene M. *H-2*-restricted cytotoxic effectors generated in vitro by the addition of trinitrophenyl-conjugated soluble proteins 352
Schreiner, George F., Cotran, Ramzi S., Pardo, Victoriano, and Unanue, Emil R. A mononuclear cell component in experimental immunological glomerulonephritis 369
Schwartz, Robert S. See Datta, Manny, Andrzejewski, André-Schwartz, and Schwartz 854
—. See Datta, McConahey, Manny, Theofilopoulos, Dixon, and Schwartz 872
Seldin, Michael F., and Rich, Robert R. Human immune responses to hapten-conjugated cells. I. Primary and secondary proliferative responses in vitro 1671
Sensenbrenner, L. L. See Volf, Sensenbrenner, Sharkis, Elfenbein, and Scher 940
Seybold, Marjorie E. See Lennon, Seybold, Lindstrom, Cochrane, and Ulevitch 973
Sharkis, S. J. See Volf, Sensenbrenner, Sharkis, Elfenbein, and Scher 940
Shearer, Gene M. See Schmitt-Verhulst, Pettinelli, Henkart, Lunney, and Shearer 352
Shek, Pang N., and Coons, Albert H. Effect of colchicine on the antibody response. I. Enhancement of antibody formation in mice 1213
—, Waltenbaugh, Carl, and Coons, Albert H. Effect of colchicine on the antibody response. II. Demonstration of the inactivation of suppressor cell activities by colchicine 1228
Shen, F. W. See Eardley, Hugenberger, McVay-Boudreau, Shen, Gershon, and Cantor 1106
Shen, F. W. See Cantor, McVay-Boudreau, Hugenberger, Naidorf, Shen, and Gershon 1116
Sherman, Linda. See Mescher, Sherman, Lemonnier, and Burakoff 946
Sherr, David H., Szewczuk, Myron R., and Siskind, Gregory W. Ontogeny of B-lymphocyte function. V. Thymus cell involvement in the functional maturation of B-lymphocytes from fetal mice transferred into adult irradiated hosts 196
Sieckmann, Donna G., Asofsky, Richard, Mosier, Donald E., Zitron, Ian M., and Paul, William E. Activation of mouse lymphocytes by anti-immunoglobulin. I. Parameters of the proliferative response 814
Sigal, Nolan H. See Cancro, Sigal, and Klinman 1
Siiteri, P. K. See Roubinian, Talal, Greenspan, Goodman, and Siiteri 1568
Simonson, Janice M. See O'Brien and Simonson 219
Simpson, Elizabeth. See Hurme, Hetherington, Chandler, and Simpson 758
—. See Hurme, Chandler, Hetherington, and Simpson 768
Singer, Alfred, Cowing, Carol, Hathcock, Karen S., Dickler, Howard B., and Hodes, Richard J. Cellular and genetic control of antibody responses in vitro. III. Immune response gene regulation of accessory cell function of non-T, non-B spleen-adherent cells 1611
Singer, S. J. See Bourguignon, Hyman, Trowbridge, and Singer 1348
Siskind, Gregory W. See Sherr, Szewczuk, and Siskind 196
Slavin, S., Fuks, Z., Kaplan, H. S., and Strober, S. Transplantation of allogeneic bone marrow without graft-versus-host disease using total lymphoid irradiation 963
—, Reitz, B., Bieber, C. P., Kaplan, H. S., and Strober, S. Transplantation tolerance in adult rats using total lymphoid irradiation: permanent survival of skin, heart, and marrow allografts 700
Smithers, S. R. See Ramalho-Pinto, McLaren, and Smithers 147
Snippe, H. See Merchant, Snippe, Lizzio, and Inman 1755
Snyder, Harry W., Jr. See Obata, Stockert, O'Donnell, Okubo, Snyder, and Old 1089
Sprent, J. Restricted helper function of F_1 hybrid T cells positively selected to heterologous erythrocytes in irradiated parental strain mice. I. Failure to collaborate with B cells of the opposite parental strain not associated with active suppression 1142
—. Restricted helper function of F_1 hybrid T cells positively selected to heterologous erythrocytes in irradiated parental strain

mice. II. Evidence for restrictions affecting helper cell induction and T-B collaboration, both mapping to the *K*-end of the *H-2* complex 1159

Sprent, J. Restricted helper function of F_1 → parent bone marrow chimeras controlled by *K*-end and of *H-2* complex 1838

Starzinski-Powitz, Anna. See Wagner, Starzinski-Powitz, Röllinghoff, Golstein, and Jakob 251

Steinberg, A. D. See Raveché, Steinberg, Klassen, and Tjio 1487

Steinman, Ralph M., Blumencranz, Steven J., Machtinger, Beverly G., Fried, Jerrold, and Cohn, Zanvil A. Mouse spleen lymphoblasts generated in vitro. Their replication and differentiation in vitro 297

—, Machtinger, Beverly G., Fried, Jerrold, and Cohn, Zanvil A. Mouse spleen lymphoblasts generated in vitro. Recovery in high yield and purity after floatation in dense bovine plasma albumin solutions 279

Stenman, Svante, and Vaheri, Antti. Distribution of a major connective tissue protein, fibronectin, in normal human tissues 1054

Stewart, Carleton C. See van der Zeijst, Stewart, and Schlesinger 1253

Stockert, Elisabeth. See Obata, Stockert, O'Donnell, Okubo, Snyder, and Old 1089

Streilein, J. W. See Zinkernagel, Callahan, Althage, Cooper, Streilein, and Klein 897

Strober, S. See Slavin, Fuks, Kaplan, and Strober 963

—. See Slavin, Reitz, Bieber, Kaplan, and Strober 700

—. See Zan-Bar, Vitetta, Assisi, and Strober 1374

Stutman, O. See Calkins, and Stutman 87

Sundharadas, G. See Narayanan and Sundharadas 1355

Swierkosz, James E., Rock, Kenneth, Marrack, Philippa, and Kappler, John W. The role of *H-2*-linked genes in helper T-cell function. II. Isolation on antigen-pulsed macrophages of two separate populations of F_1 helper T cells each specific for antigen and one set of parental *H-2* products 554

Sy, Man-Sun. See Miller, Sy, and Claman 788

Szewczuk, Myron R. See Sherr, Szewczuk, and Siskind 196

T

Tada, Tomio, Takemori, Toshitada, Okumura, Ko, Nonaka, Makoto, and Tokuhisa, Takeshi. Two distinct types of helper T cells involved in the secondary antibody response: independent and synergistic effects of Ia^- and Ia^+ helper T cells 446

Takemori, Toshitada. See Tada, Takemori, Okumura, Nonaka, and Tokuhisa 446

Talal, N. See Roubinian, Talal, Greenspan, Goodman, and Siiteri 1568

Tan, Eng M. See Alspaugh, Jensen, Rabin, and Tan 1018

—. See Eisenberg, Tan, and Dixon 582

Targan, Stephan. See Jondal and Targan 1621

Tashjian, Armen H., Jr. See Voelkel, Levine, Alper, and Tashjian 1078

Theofilopoulos, Argyrios N. See Datta, McConahey, Manny, Theofilopoulos, Dixon, and Schwartz 872

Thèze, Jacques. See Germain, Thèze, Kapp, and Benacerraf 123

Thomson, Napier M. See Holdsworth, Thomson, Glasgow, Dowling, and Atkins 98

Thompson, Russell E., Mandle, Robert, Jr., and Kaplan, Allen P. Characterization of human high molecular weight kininogen. Procoagulant activity associated with the light chain of kinin-free high molecular weight kininogen 488

Tjio, J. H. See Raveché, Steinberg, Klassen, and Tjio 1487

Tokuhisa, Takeshi. See Tada, Takemori, Okumura, Nonaka, and Tokuhisa 446

Trang, Hoang T. K. See Rabellino, Ross, Trang, Williams, and Metcalf 434

Trinchieri, Giorgio, Santoli, Daniela, Dee, Robert R., and Knowles, Barbara B. Antiviral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Identification of the anti-viral activity as interferon and characterization of the human effector lymphocyte subpopulation 1299

—, and Santoli, Daniela. Anti-viral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Enhancement of human natural killer cell activity by interferon and antagonistic inhibition of susceptibility of target cells to lysis 1314

Trowbridge, Ian. See Bourguignon, Hyman, Trowbridge, and Singer 1348

Tung, Jwu-Sheng, O'Donnell, Paul V., Fleissner, Erwin, and Boyse, Edward A. Relationships of gp70 of MuLV envelopes to gp70 components of mouse lymphocyte plasma membranes 1280

U

Udem, Stephen. See Ju, Udem, Rager-Zisman, and Bloom 1637

Ulevitch, Richard. See Lennon, Seybold, Lindstrom, Cochrane, and Ulevitch 973

Unanue, Emil R. See Schreiner, Cotran, Pardo, and Unanue 369

V

Vaerman, Jean-Pierre. See Lemaitre-Coelho, Jackson, and Vaerman 934

Vaheri, Antti. See Stenman and Vaheri 1054

van der Loo, Wessel. See Black, van der Loo, Loken, and Herzenberg 984

van der Meer, Jos W. M., Bulterman, Dini, van Zwet, Theda L., Elzenga-Claasen, Ine, and van Furth, Ralph. Culture of mononuclear phagocytes on a teflon surface to prevent adherence 271

van der Zeijst, Ben A. M., Stewart, Carleton, C., and Schlesinger, Sondra. Proliferative capacity of mouse peritoneal macrophages in vitro 1253

van Furth, Ralph. See van der Meer, Bulterman, van Zwet, Elzenga-Claasen, and van Furth 271

van Zwet, Theda L. See van der Meer, Bulterman, van Zwet, Elzenga-Claasen, and van Furth 271

Vassalli, Jean-Dominique, Granelli-Piperno, Angela, Griscelli, Claude, and Reich, E. Specific protease deficiency in polymorphonuclear leukocytes of Chédiak-Higashi syndrome and beige mice 1285

Verbonitz, Martha W., Ennis, Francis A., Hicks, John T., and Albrecht, Paul. Hemagglutinin-specific complement-dependent cytolytic antibody response to influenza infection 265

Vitetta, E. S. See Zan-Bar, Vitetta, Assisi, and Strober 1374

Voelkel, Edward F., Levine, Lawrence, Alper, Chester A., and Tashjian, Armen H., Jr. Acute phase reactants ceruloplasmin and haptoglobin and their relationship to plasma prostaglandins in rabbits bearing the VX$_2$ carcinoma 1078

Volf, D., Sensenbrenner, L. L., Sharkis, S. J., Elfenbein, G. J., and Scher, I. Induction of partial chimerism in nonirradiated B-lymphocyte-deficient CBA/N mice 940

von Boehmer, Harald, Haas, Werner, and Pohlit, Helmut. Cytotoxic T cells recognize male antigen and H-2 as distinct entities 1291

W

Wagner, Hermann, Starzinski-Powitz, Anna, Röllinghoff, Martin, Golstein, Pierre, and Jakob, Hedwig. T-cell-mediated cytotoxic immune responses to F9 teratocarcinoma cells: cytolytic effector T cells lyse H-2-negative F9 cells and syngeneic spermatogonia 251

Wagshal, Alan B., Jegasothy, Brian V., and Waksman, Byron H. Regulatory substances produced by lymphocytes. VI. Cell cycle specificity of inhibitor of DNA synthesis action in L cells 171

Waksman, Byron H. See Wagshal, Jegasothy, and Waksman 171

Waltenbaugh, Carl. See Shek, Waltenbaugh, and Coons 1228

Warner, Carol M., Berntson, Tom J., Eakley, Lois, McIvor, Judith L., and Newton, Robert C. The immune response of allophenic mice to DNP$_{56}$ BGG. I. Allotype analysis of anti-dinitrophenyl antibody 1849

Watson, James, Largen, Michael, and McAdam, Keith P. W. J. Genetic control of endotoxic responses in mice 39

Webb, Susan R. See Grossi, Webb, Zicca, Lydyard, Moretta, Mingari, and Cooper 1405

Weigle, William O. See Goodman, Parks, and Weigle 800

Weiler, John M., Yurt, Roger W., Fearon, Douglas T., and Austen, K. Frank. Modulation of the formation of the amplification convertase of complement, C3b, Bb, by native and commercial heparin 409

Weiss, Stephen J., Rustagi, Pradip K., and LoBuglio, Albert F. Human granulocyte generation of hydroxyl radical 316

Wekerle, Hartmut. Immunological T-cell memory in the in vitro-induced experimental autoimmune orchitis. Specificity of the reaction and tissue distribution of the autoantigens 233

Werb, Zena. Biochemical actions of glucocorticoids on macrophages in culture. Specific inhibition of elastase, collagenase, and plasminogen activator secretion and effects on other metabolic functions 1695

—, Foley, Rosemary, and Munck, Allan. Interaction of glucocorticoids with macrophages. Identification of glucocorticoid receptors in monocytes and macrophages 1684

Wettstein, Peter J., Bailey, Donald W., Mobraaten, Larry E., Klein, Jan, and Frelinger, Jeffrey A. T-lymphocyte response to *H-2* mutants. I. Proliferation is dependent on Ly 1$^+$2$^+$ cells 1395

Wight, E. See Aguet, Andersson, Andersson, Wight, Binz, and Wigzell 50

Wigzell, Hans. See Aguet, Andersson, Andersson, Wight, Binz, and Wigzell 50

—. See Binz, and Wigzell 63

—. See Frischknecht, Binz, and Wigzell 500

—. See Janeway, Murphy, Kemp, and Wigzell 1065

—. See Kimura and Wigzell 1418

—. See Peck, Murgita, and Wigzell 667

Williams, Neil. See Rabellino, Ross, Trang, Williams, and Metcalf 434

Winchester, Robert J. See Ross, Jarowski, Rabellino, and Winchester 730

Y

Yap, K. L. See Braciale and Yap 1236

Yurt, Roger W. See Weiler, Yurt, Fearon, and Austen 409

Z

Zan-Bar, I., Vitetta, E. S., Assisi, F., and Strober, S. The relationship between surface immunoglobulin isotype and immune function of murine B lymphocytes. III. Expression of a single predominant isotype on primed and unprimed B cells 1374

Zarling, Joyce M., and Bach, Fritz H. Sensitization of lymphocytes against pooled allogeneic cells. I. Generation of cytetoxicity against autologous human lymphoblastoid cell lines 1334

Zicca, Antonio. See Grossi, Webb, Zicca, Lydyard, Moretta, Mingari, and Cooper 1405

Ziccardi, Robert J. and Cooper, Neil R. Demonstration and quantitation of activation of the first component of complement in human serum 385

Ziegler, Elizabeth J. See Davis, Ziegler, and Arnold 1007

Ziff, M. See Botzenhardt, Klein, and Ziff 1435

Zinkernagel, R. M., Callahan, G. N., Althage, A., Cooper, S., Klein, P. A., and Klein, J. On the thymus in the differentiation of "H-2 self-recognition" by T cells: evidence for dual recognition? 882

—, Callahan, G. N., Althage, A., Cooper, S., Streilein, J. W., and Klein, J. The lymphoreticular system in triggering virus plus self-specific cytotoxic T cells: evidence for T help 897

Zitron, Ian M. See Sieckmann, Asofsky, Mosier, Zitron, and Paul 814

INDEX TO SUBJECTS

A

Abelson virus-induced leukemia, effect of pseudotype, 1044
– virus-induced transformation, lymphoid cells, effect of helper virus, 1126
Accessory cell function, non-T, non-B spleen adherent cells, immune response gene regulation, 1611
Adherence, prevention by culture of mononuclear phagocytes on Teflon surface, 271
Agglutinin, A, insolubilized, *Dolichos biflorus* competitive binding assays of A_1 and A_2 blood groups, 830
Aggregates, IgG, and immune complexes, platelet response to, effect of lipid A-rich bacterial lipopolysaccharides, 207
Aging, effect of proliferative capacity, immunohemopoietic stem cells, 1526
L-Alanine^{30}-L-tyrosine^{10}, L-glutamic acid^{60}-, nonresponder mice primed with, lymphoid cell immunosuppression, 997
–, L-glutamic acid^{60}-, specific suppressor T cells, induction of, antigen-specific suppressor factor, 123
Albumin, bovine, flotation of mouse spleen lymphoblasts, 279
–, transferrin, and soybean lipid, replacement of serum, cultures of lipopolysaccharide-reactive B lymphocytes, 923
Alkaline phosphodiesterase I, mouse peritoneal macrophage plasma membrane, 77
Alloantigens, and anti-idiotypic antibodies, binding to same receptors, activated T cells, 25
–, derepressed, lung tumor-associated, coded by *K* region of *H-2* major histocompatibility complex, 1363
–, lymphocyte, migration with specific reactivity to, 13
–, stimulating, suppressor T cell specific for, mixed lymphocyte reaction in man, 137
Alloantisera, anti-Ia, I-J controlled determinants, in vivo elimination of specific suppression, 656
Allogeneic bone marrow transplantation, without graft-versus-host disease, total lymphoid irradiation, 963
– cells, lymphocyte sensitization, generation of cytotoxicity against autologous human lymphoblastoid cell lines, 1334
– effect factor, *H-2*-restricted, B-cell-derived, *I*-region control of during B-cell activation, 1198
Allografts, skin, mouse, effects of anti-H-2K, H-2D, and anti-Ia antisera, 611
Allophenic mice, immune response to DNP_{56}BGG, allotype analysis of anti-DNP antibody, 1849
Allotype analysis, anti-DNP antibody, immune response of allophenic mice to DNP_{56}BGG, 1849
Alternative pathway components, effect on β1H globulin binding to cell-bound C3b, 1792
Amastigote multiplication, hamster macrophages, leishmaniasis, 515
Amplification convertase of complement, C3b, Bb, effect of heparin, 409
Anti-A serum, insolubilized, *Dolichos biflorus* competitive binding assays of A_1 and A_2 blood groups, 830
Antibodies, anti-idiotypic, and alloantigens, binding to same receptors, activated T cells, 25
–, anti-idiotypic, induction of transplantation immune reactions, 500
–, anti-nucleic acid, NZB/NZW F_1 mice, effect of castration and sex hormones, 1568
–, staphylococcal nuclease, gene mapping for, 1517
Antibody, anti-DNP, allotype analysis, immune response of allophenic mice to DNP_{56}BGG, 1849
–, anti-nuclease, staphylococcal, role of non-*H-2*-linked genes in control of, 396
–, core glycolipid, neutralization of meningococcal endotoxin, 1007
–, cytolytic response, hemagglutinin-specific complement-dependent, influenza infection, 265
– formation, mouse, effect of colchicine, 1213
– response, colchicine inactivation of suppressor cell activity, 1228
– response, genetic control, accessory cell function of non-T, non-B spleen adherent cells, 1611
– response, secondary, Ia^- and Ia^+ helper T cells, 446
Antibody-dependent cellular cytotoxicity, allosensitized human T cells, 605
Antigen, auto-, distribution, immunological T-cell memory, autoimmune orchitis, 233
–, cell surface, $\text{G}_{(\text{RADA1})}$, mouse leukemia, relationship to murine leukemia virus, 1089
–, male, and *H-2*, recognition by cytotoxic T cells, 1291
–, nuclear, induction of, rheumatoid arthritis, lymphocyte transformation by Epstein-Barr virus, 1018

Antigen, requirement, induction of B memory cells 1824
–, specific, regulation of macrophage plasminogen activator, Bacille Calmette-Guérin infection, mouse, 1175
Antigen-pulsed macrophages, helper T cells, specific for antigen and parental *H-2* products, 554
Antigens, allo-, and anti-idiotypic antibodies, binding to same receptors, activated T cells, 25
–, allo-, derepressed, lung tumor, coded by *K* region of *H-2* major histocompatibility complex, 1363
–, allo-, lymphocyte migration with specific reactivity to, 13
–, differentiation, Ly 1, Ly 2, Thy 1, and TL, mouse T-cell tumors, 1267
–, *H-2*, thymocytes, radiation leukemia virus infection, 470
–, *H-2D*, self and non-self, cytotoxic T-cell responses, 1661
–, *H-2K* and *H-2D*, nonrandom inclusion of in Friend virus particles, 340
–, Ia-like, complement receptors, appearance during human neutrophil maturation, 730
Antigen-specific suppressor factor, target and genetics of, 110
– T-cell-suppressor factor and antigen, induction of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} specific suppressor T cells, 123
Anti-H-2K, H-2D, and anti-Ia antisera, effects on murine skin allografts, 611
Anti-Ia alloantisera, I-J controlled determinants, in vivo elimination of specific suppression, 656
Anti-Ia-associated proliferation, inhibition of, immunoregulatory activity of α-fetoprotein, 667
Anti-immunoglobulin activation of mouse lymphocytes, 814
Anti-phagocytosis, streptococcal M protein, correlation of immunological cross-reactions and structure, 1771
Antisera, anti-H-2K, H-2D, and anti-Ia, effects on murine skin allografts, 611
Anti-Sm reactivity, autoimmune mouse strains, 582
Anti-viral activity, interferon, characterization of human effector lymphocyte subpopulation, 1299
– activity, interferon, enhancement of human natural killer cell activity, 1314
Athymic mice, bone marrow cells, thymosin induction of deoxynucleotidyl transferase, 708
Autoantibody, spontaneous production of, genetic studies in NZB mice, 1487
Autoantigen distribution, immunological T-cell memory, autoimmune orchitis, 233
Autoanti-idiotypic killer T cells, lymphocyte transfer of suppression to normal syngeneic recipients, 63
Autoimmune mouse strains, anti-Sm reactivity, 582
– myasthenia gravis, role of complement in pathogenesis of, 973
Autoimmunity and retrovirus expression, New Zealand black mice, viral envelope glycoprotein gp70, 872
– and retrovirus expression, New Zealand black mice, xenotropic virus, 854
Autoimmunogen, mixed leukocyte culture-activated T lymphoblasts, specific immune unresponsiveness, cellular and serological effects, 50

B

B cells, immune response genes for poly(L,Tyr,Glu)-poly-DL-Ala-L-Lys, 1596
– cells, memory, antigen requirements for induction of, 1824
– cells, memory, maturation of, loss of surface IgD, 984
– cells, noncollaboration with F$_1$ hybrid T cells of opposite parental strain, 1142
B lymphocyte functional maturation, role of thymus, 196
– lymphocyte-deficient nonirradiated CBA/N mice, partial chimerism, 940
– lymphocytes, lipopolysaccharide-reactive, cultures of, serum replacement by albumin, transferrin, and soybean lipid, 923
– lymphocytes, murine, surface immunoglobulin isotype and immune function, 1374
Bacille Calmette-Guérin infection, mouse, regulation of macrophage plasminogen activator by T lymphocytes and specific antigen, 1175
Bacterial lipopolysaccharides, lipid A-rich, effect on platelet response to immune complexes and IgG aggregates, 207
B-cell activation, *I*-region control of B-cell-derived *H-2*-restricted allogeneic effect factor and receptor during, 1198
– blockade, X-linked hapten-specific, CBA/N mice, 1755
– clones, mitogen-stimulated, switch from IgM to IgG secretion, 1744
– primary repertoire, influenza-specific, BALB/c mice, 776
Beige mice, protease deficiency in polymorphonuclear leukocytes, 1285
Bile duct obstruction, rat, secretory IgA and free secretory component in serum, 934
– immunoglobin A, active transport from blood, 588
Binding assays, competitive, A$_1$ and A$_2$ blood groups with insolubilized anti-A serum and A agglutinin from *Dolichos biflorus*, 830
–, β1H globulin to cell-bound C3b, effect of alternative pathway components, 1792
Blood, immunoglobin A, active transport to bile, 588
– groups A$_1$ and A$_2$, competitive binding, in-

solubilized anti-A serum and A agglutinin from *Dolichos biflorus*, 830
Bone marrow, allogeneic, transplantation without graft-versus-host disease, total lymphoid irradiation, 963
– marrow cells, athymic mice, thymosin induction of deoxynucleotidyl transferase, 708
– marrow chimeras, restricted helper function controlled by *K*-end of *H-2* complex, 1838
Burst-forming unit, erythroid, human, T-cell in vitro proliferation, 324
Butanol-extracted lipopolysaccharide, immunologic responsiveness to, C3H/HeJ mouse, 800
Bvr-1, restriction locus, endogenous BALB virus, cat × mouse somatic cell hybrids, 219

C

Carcinoma, VX_2, rabbits, relationship of ceruloplasmin and haptoglobin to plasma prostaglandins, 1078
Castration, effect on survival, anti-nucleic acid antibodies, and glomerulonephritis, NZB/NZW mice, 1568
Cat × mouse somatic cell hybrids, pleiotropic restriction of endogenous BALB virus, *Bvr-1* locus, 219
Cell cycle specificity, lymphocyte inhibitor of DNA synthesis, L cells, 171
– entry, hamster macrophages, leishmaniasis, 515
– surface glycoprotein, murine cytotoxic T lymphocytes, 1418
Cell-mediated lympholysis, against syngeneic targets at major histocompatibility complex, NZB mice, 1435
Cells, B, primary repertoire, influenza-specific, BALB/c mice, 776
–, bone marrow, athymic mice, thymosin induction of deoxynucleotidyl transferase, 708
–, cytotoxic effector, induction of spontaneous killer cell specificity, 1621
–, epithelial, secretory component, surface receptor for polymeric immunoglobulins, 1832
–, hapten-conjugated, human, immune proliferative responses to, 1671
–, helper T, Ia^- and Ia^+, secondary antibody response, 446
–, human endothelial, fibronectin synthesis, 1779
–, L, DNA synthesis, cell cycle specificity of lymphocyte inhibitor, 171
–, L, and macrophages, interaction with *Pneumocystis carinii*, 157
–, lymphoid, Abelson virus-induced transformation, effect of helper virus, 1126
–, memory B, maturation of, loss of surface IgD, 984
–, mutant Thy-1^-, localization of T25 glycoprotein by immunofluorescence and immunoelectron microscopy, 1348
Cells, neoplastic, cultured, serine enzymes, 745
–, phagocytic, chemiluminescence induced by *N*-formylmethionyl peptides, 182
–, stem, immunohemopoietic, loss of proliferative capacity, effects of aging and transplantation, 1526
–, T, activated, binding of alloantigens and anti-idiotypic antibodies to same receptors, 25
–, T, cytolysis, hexose requirement at lethal hit stage, 1551
–, T, cytotoxic, influenza virus-specific, role of viral infectivity in induction of, 1236
–, T, cytotoxic, recognition of male antigen and *H-2*, 1291
–, T, cytotoxic responses to H-Y, Ir gene mapping, 758
–, T, cytotoxic responses to H-Y, rejection of syngeneic male skin grafts, 768
–, T, cytotoxic, self-specific, plus virus, lymphoreticular system triggering of, role of thymus, 897
–, T, differentiation of *H-2* self-recognition, thymus, 882
–, T, F_1 hybrid, selected to heterologous erythrocytes, helper function, noncollaboration with B cells of opposite parental strain, 1142
–, T, F_1 hybrid, selected to heterologous erythrocytes, helper function, T-B collaboration, mapping to K-end of *H-2* complex, 1159
–, T, human, allosensitized, antibody-dependent cellular cytotoxicity, 605
–, T, lymphocyte-activating determinant locus, mapping in *I-C* subregion, 1028
–, T, specific for hapten-modified self, precommitment for self major histocompatibility complex antigens, 1065
–, T, subpopulations, human, receptors for IgM or IgG, 1405
–, T, suppressor, specific for stimulating alloantigen, mixed lymphocyte reaction, in man, 137
–, teratocarcinoma F9, T-cell-mediated cytotoxic immune response to, 251
Cellular restrictions, immunoregulatory activity of α-fetoprotein, inhibition of anti-Ia-associated proliferation, 667
Ceruloplasmin, relationship to plasma prostaglandins, VX_2 carcinoma in rabbits, 1078
Chédiak-Higashi syndrome, protease deficiency in polymorphonuclear leukocytes, 1285
Chemiluminescence of phagocytic cells, *N*-formylmethionyl peptides, 182
Chimera, bg^J/bg^+:W/W^v, hematopoietic stem cell regulatory volumes, 1189
–, bone marrow, restricted helper function controlled by *K* end of *H-2* complex, 1838
–, partial, nonirradiated B lymphocyte-deficient CBA/N mice, 940

Clones, B-cell, mitogen-stimulated, switch from IgM to IgG secretion, 1744

Clonotype, equivalent, differential expression in BALB/c and C57BL/6 mice, 1

Colchicine, effect on antibody response, inactivation of suppressor cell activity, 1228

–, enhancement of mouse antibody formation, 1213

Collagenase secretion, cultured macrophages, glucocorticoid inhibition of, 1695

Collagens, fibronectin affinity to, 1584

Complement, C3b,Bb, effect of heparin on amplification convertase of, 409

–, cell-bound C3b, binding of β1H globulin, effect of alternative pathway components, 1792

–, human serum, activation of first component, 385

– receptor, p15E, oncornavirus lysis by human serum, 844

– receptors, Ia-like antigens, appearance during human neutrophil maturation, 730

–, role in eosinophil killing of schistosomula of *Schistosoma mansoni*, 147

–, role in hemagglutinin-specific cytolytic antibody response to influenza infection, 265

–, role in pathogenesis of autoimmune myasthenia gravis, 973

–, role in thrombin-induced platelet function, 1713

Component, first, of human serum complement, activation of, 385

Connective tissue protein, fibronectin, distribution in normal human tissues, 1054

Contact activation of plasma, surface and fluid phase activities of activated Hageman factor, 719

– sensitivity, 1-fluoro-2,4-dinitrobenzene, tolerance to, lymphoid cell induction of suppressor T cells, role of *H-2D* region of major histocompatibility complex, 788

Core glycolipid, antibody to, neutralization of meningococcal endotoxin, 1007

Crescentic glomerulonephritis, tissue culture of isolated glomeruli, 98

–, T-cell-mediated, hexose requirement at lethal hit stage, 1551

Cytolytic T lymphocytes, secondary, induction by solubilized membrane proteins, 946

Cytotoxic effector cells, induction of spontaneous killer cell specificity, 1621

– effectors, *H-2* restricted, induction by trinitrophenyl-conjugated soluble proteins, 352

– immune responses, T-cell-mediated, lysis of *H-2*-negative F9 cells and syngeneic spermatogonia, 251

– T cells, influenza virus-specific, role of viral infectivity in induction of, 1236

– T cells, recognition of male antigen and *H-2*, 1291

– T cells, self-specific, plus virus, lymphoreticular system triggering of, role of thymus, 897

Cytotoxic T lymphocytes, murine, cell surface glycoproteins, 1418

– T-cell responses to H-Y, Ir gene mapping, 758

– T-cell responses to H-Y, rejection of syngeneic male skin grafts, 768

– T-cell responses, self and non-self *H-2D* antigens, restricted specificity, 1661

Cytotoxicity, cellular, antibody-dependent, allosensitized human T cells, 605

–, generation of against autologous human lymphoblastoid cell lines, 1334

–, lympho-, T-cell-mediated, leukemia-specific, acute myelogenous leukemia, 912

–, T lymphocytes, effects of polyadenylic: polyuridylic acid and lipopolysaccharide on generation of, 1355

D

Deoxynucleotidyl transferase, induction by thymosin, bone marrow cells from athymic mice, 708

Determinants, I-J, in vivo elimination of specific suppression, anti-Ia alloantisera, 656

Dextran B512, immunological unresponsiveness, lack of Ig receptor expression, 645

Differentiation, induction systems, generation and regulation of lymphocyte populations, 1727

–, mouse leukocytes, membrane receptors and phagocytic capacity, 434

– and replication in vitro, mouse spleen lymphoblasts, 297

DNA coupled to T-dependent and T-independent carriers, induction of B-memory cells, 1824

–, single-stranded, circulating, removal of, in mice, 684

– synthesis, lymphocyte inhibitor of, L cells, cell cycle specificity, 171

DNP$_{56}$BGG, immune response to, allophenic mice, allotype analysis of anti-DNP antibody, 1849

Dolichos biflorus, insolubilized anti-A serum and A agglutinin, competitive binding assays of A$_1$ and A$_2$ blood groups, 830

E

Effector cells, cytotoxic, induction of spontaneous killer cell specificity, 1621

Effectors, *H-2* restricted cytotoxic, induction by trinitrophenyl-conjugated soluble proteins, 352

Elastase secretion, cultured macrophages, glucocorticoid inhibition of, 1695

Endothelial cells, human, fibronectin synthesis, 1779

Endotoxic responses, mouse, genetic control, 39

Endotoxin, meningococcal, neutralization by antibody to core glycolipid, 1007
Energy and hexose requirement, lethal hit stage, T-cell-mediated cytolysis, 1551
Envelopes, MuLV, gp70 components of mouse lymphocyte plasma membrane, 1280
–, viral, glycoprotein gp70, autoimmunity and retrovirus expression, New Zealand black mice, 872
Enzymes, serine, cultured neoplastic cells, 745
Eosinophil, rat, killing of *Schistosoma mansoni* schistosomula, complement-mediated, 147
Epithelial cell secretory components, surface receptor for polymeric immunoglobulins, 1832
Epstein-Barr virus, lymphocyte transformation, induction of nuclear antigen, rheumatoid arthritis, 1018
Erythrocytes, heterologous, F_1 hybrid T cells selected to, helper function, noncollaboration with B cells of opposite parental strain, 1142
–, heterologous, F_1 hybrid T cells selected to, helper function, T-B collaboration, mapping to K-end of *H-2* complex, 1159
–, IgG-coated, effect of interferon inducers on macrophage ingestion of, 593
Erythroid burst-forming unit, human, T-cell in vitro proliferation, 324

F

Factor, migration inhibitory, human, 541
–, suppressor, antigen-specific, target and genetics of, 110
–, suppressor, T-cell-, antigen-specific, induction of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} specific suppressor T cells, 123
–, T-cell, soluble, suppression of mixed lymphocyte reaction, in man, 1037
Feedback inhibition, immunoregulatory circuits, T-cell sets, absence in NZB mice, 1116
– inhibition, immunoregulatory circuits, T-cell sets, role of T-helper cells, 1106
α-Fetoprotein, immunoregulatory activity, inhibition of anti-Ia-associated proliferation, 667
Fibrinogen, fibronectin affinity to, 1584
Fibronectin affinity to collagens and fibrinogen, 1584
–, connective tissue protein, distribution in normal human tissues, 1054
– synthesis, human endothelial cells, 1779
Fluid phase activity, activated Hageman factor, contact activation of plasma, 719
1-Fluoro-2,4-dinitrobenzene contact sensitivity, tolerance to, lymphoid cell induction of suppressor T cells, role of *H-2D* region of major histocompatibility complex, 788
N-Formylmethionyl peptides, induction of chemiluminescence, phagocytic cells, 182
Friend virus leukemia, genetic resistance to, gene (*Fv-3*) regulating immunosuppression, 422
Friend virus particles, nonrandom inclusion of *H-2K* and *H-2D* antigens, 340

G

$G_{(\mathrm{RADA1})}$, cell surface antigen, mouse leukemia, relationship to murine leukemia virus, 1089
Gene mapping for anti-nuclease idiotypes, genetic control of immune response to staphylococcal nuclease, 1517
– mapping, Ir, cytotoxic T-cell responses to H-Y, 758
Genes, *H-2*-linked, role in helper T-cell function, antigen-pulsed macrophages specific for antigen and parental *H-2* products, 554
–, *H-2* linked, role in helper T-cell function, immune response for poly (L-Tyr,Glu)-poly-DL-Ala-poly-L-Lys in B cells and macrophages, 1596
–, lupus nephritis, NZB × NZW mice, 1653
–, V, nonrandom expression, immunological unresponsiveness to dextran B512, 645
Genetic basis, susceptibility to mouse leukemia induction by 3-methylcholanthrene, 459
– control of antibody responses, accessory cell function of non-T, non-B spleen adherent cells, 1611
– control, endotoxic responses in mice, 39
– control, immune response to staphylococcal nuclease, mapping of genes for anti-nuclease idiotypes, 1517
– control, immune response to staphylococcal nuclease, role of non-*H-2*-linked genes in anti-nuclease antibody response, 396
– relationships, antigen-specific suppressor factor, 110
– resistance to Friend virus leukemia, gene (*Fv-3*) regulating immunosuppression, 422
– restrictions, immunoregulatory activity of α-fetoprotein, inhibition of anti-Ia-associated proliferation, 667
– restrictions, suppressor T-cell induction by lymphoid cells, tolerance to 1-fluoro-2,4-dinitrobenzene contact sensitivity, role of *H-2D* region of major histocompatibility complex, 788
– studies, autoimmunity and retrovirus expression, New Zealand black mice, viral envelope glycoprotein gp70, 872
– studies, autoimmunity and retrovirus expression, New Zealand black mice, xenotropic virus, 854
– studies, NZB mice, spontaneous autoantibody production, 1487
β1H Globulin binding to cell-bound C3b, effect of alternative pathway components, 1792
Glomerulonephritis, crescentic, tissue culture of isolated glomeruli, 98
–, immunological, mononuclear cell component, 369

Macrophage plasminogen activator, regulation by T lymphocytes and specific antigen, Bacille Calmette-Guérin infection, mouse, 1175
Macrophages, antigen-pulsed, helper T cells, specific for antigen and parental *H-2* products, 554
–, cultured, glucocorticoid inhibition of elastase, collagenase, and plasminogen activator secretion, 1695
–, hamster, leishmaniasis, cell entry, intracellular survival, amastigote multiplication, 515
–, immune response genes for poly-(L-Tyr,Glu)-poly-DL-Ala-poly-L-Lys, 1596
– and L cells, interaction with *Pneumocystis carinii*, 157
– and monocytes, glucocorticoid receptors, 1684
–, peritoneal, mouse, in vitro proliferative capacity, 1253
–, peritoneal, mouse, plasma membrane alkaline phosphodiesterase I, 77
–, peritoneal, mouse, prostaglandin E production, 952
Male antigen and *H-2*, recognition by cytotoxic T cells, 1291
–, syngeneic, skin graft rejection, cytotoxic T-cell responses to H-Y, 768
Man, activation of first component of complement, 385
–, autologous lymphoblastoid cell lines, generation of cytotoxicity against, 1334
–, blood monocytes, prostaglandin E production, 952
–, effector lymphocyte subpopulation, interferon anti-viral activity, 1299
–, endothelial cells, fibronectin synthesis, 1779
–, erythroid burst-forming unit, T-cell proliferation in vitro, 324
–, granulocyte generation of hydroxyl radical, 316
–, high molecular weight kininogen, procoagulant activity of light chain of, 488
–, immune proliferative responses to hapten-conjugated cells, 1671
–, lymphoblastoid cell lines, temperature-sensitive measles virus mutants, 1637
–, lymphocyte stimulation by mitogens, inhibition by potassium ionophore, 571
–, migration inhibitory factor, 541
–, mixed lymphocyte reaction, suppressor T-cell specific for stimulating alloantigen, 137
–, natural killer cell activity, enhancement by interferon, 1314
–, neutrophils, appearance of Ia-like antigens and complement receptors during maturation of, 730
–, serum, lysis of oncornavirus, identification of complement (C1) receptor as p15E, 844
–, suppression of mixed lymphocyte reaction by soluble T-cell factor, 1037
–, T-cell subpopulations, receptors for IgM or IgG, 1405
Man, T cells, allosensitized, antibody-dependent cellular cytotoxicity, 605
Mapping, Ir genes, cytotoxic T-cell responses to H-Y, 758
–, *K*-end of *H-2* complex, T-B collaboration, helper cell induction, 1159
Measles virus, temperature-sensitive mutants, isolation from infected human lymphoblastoid cell lines, 1637
Membrane, plasma, alkaline phosphodiesterase I, mouse peritoneal macrophages, 77
–, plasma, mouse lymphocyte, gp70 components, MuLV envelopes, 1280
– proteins, solubilized, induction of secondary cytolytic T lymphocytes, 946
– receptors, phagocytic capacity, mouse leukocyte differentiation, 434
Memory B cells, maturation of, loss of surface IgD, 984
– cells, B, antigen requirements for induction of, 1824
–, immunological, T-cell, autoantigen distribution, autoimmune orchitis, 233
Meningococcal disease, group B, immunogenicity of serotype 2 vaccines, specificity of protection, guinea pig model, 629
– disease, group B, infection and resulting immunity, guinea pig model, 619
– endotoxin, neutralization by antibody to core glycolipid, 1007
3-Methylcholanthrene, induction of mouse leukemia, genetic basis for susceptibility to, 459
Migration inhibitory factor, human, 541
–, lymphocytes with specific reactivity to alloantigens, 13
Mitogen stimulation of human lymphocytes, inhibition by potassium ionophore, 571
Mitogen-stimulated B-cell clones, switch from IgM to IgG secretion, 1744
Mixed leukocyte culture-activated T lymphoblasts, specific immune unresponsiveness, autoanti-idiotypic killer T cells, suppression transfer to normal syngeneic recipients, 63
– leukocyte culture-activated T lymphoblasts, specific immune unresponsiveness, cellular and serological effects, 50
– lymphocyte reaction, suppression by soluble T-cell factor, in man, 1037
– lymphocyte reaction, suppressor T cell specific for stimulating alloantigen, 137
Monocytes, blood, human, prostaglandin E production, 952
– and macrophages, glucocorticoid receptors, 1684
Mononuclear cell component, immunological glomerulonephritis, 369
Mouse, allophenic, immune response to DNP$_{56}$BGG, allotype analysis of anti-DNP antibody, 1849
–, antibody formation, effect of colchicine, 1213
–, athymic, bone marrow cells, thymosin in-

duction of deoxynucleotidyl transferase, 708

Mouse, autoimmune strains, anti-Sm reactivity, 582

–, Bacille Calmette-Guérin infection, regulation of macrophage plasminogen activator by T lymphocytes and specific antigen, 1175

–, BALB/c, influenza-specific primary B-cell repertoire, 776

–, BALB/c and C57BL/6, differential expression of equivalent clonotype, 1

–, beige, protease deficiency in polymorphonuclear leukocytes, 1285

– × cat somatic cell hybrids, pleiotropic restriction of endogenous BALB virus, *Bvr-1* locus, 219

–, CBA/N, nonirradiated B lymphocyte-deficient, partial chimerism, 940

–, CBA/N, X-linked, hapten-specific B-cell blockade, 1755

–, C3H/HeJ, immunologic responsiveness to butanol-extracted lipopolysaccharide, 800

–, cytotoxic T lymphocytes, cell surface glycoproteins, 1418

–, genetic resistance to Friend virus leukemia, gene (*Fv-3*) regulating immunosuppression, 422

– histiocytic and myelomonocytic tumor cell lines, in vitro pyrogen production, 1503

–, inborn resistance to myxoviruses, phenotype of macrophages, 531

–, leukemia, cell surface antigen, G$_{\text{(RADA1)}}$, relationship to murine leukemia virus, 1089

– leukemia, genetic basis of susceptibility to induction by 3-methylcholanthrene, 459

–, leukocyte differentiation, membrane receptors and phagocytic capacity, 434

– lymphocyte plasma membranes, gp70 components, MuLV envelopes, 1280

– lymphocytes, activation by anti-immunoglobulin, 814

– myeloma cell line, production of IgG$_{2a}$ variants, 1535

–, New Zealand black, autoimmunity and retrovirus expression, viral envelope glycoprotein gp70, 872

–, New Zealand black, autoimmunity and retrovirus expression, xenotropic virus, 854

–, nonresponder, primed with L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}, lymphoid cell immunosuppression, 997

–, NZB, absence of feedback inhibition, immunoregulatory circuits, T-cell sets, 1116

–, NZB, cell-mediated lympholysis against syngeneic targets, major histocompatibility complex, 1435

–, NZB, genetic studies in, spontaneous autoantibody production, 1487

–, NZB/NZW F$_1$, effect of castration and sex hormone on survival, anti-nucleic acid antibodies, and glomerulonephritis, 1568

–, NZB × NZW, three genes for lupus nephritis, 1653

Mouse, peritoneal macrophages, in vitro proliferative capacity, 1253

–, peritoneal macrophages, plasma membrane alkaline phosphodiesterase I, 77

–, peritoneal macrophages, prostaglandin E production, 952

–, postnatal development, changes in suppressor mechanisms, 87

–, removal of circulating single-stranded DNA, 684

–, skin allografts, effects of anti-H-2K, H-2D, and anti-Ia antisera, 611

–, spleen lymphoblasts, floatation in bovine albumin solutions, 279

–, spleen lymphoblasts, in vitro replication and differentiation, 297

–, T-cell tumors, Ly-1, Ly-2, Thy-1, and TL differentiation antigens, 1267

MuLV envelopes, gp70 components of mouse lymphocyte plasma membranes, 1280

Mutant, *H-2*, T lymphocyte proliferative response to, 1395

– Thy-1$^-$ cells, localization of T25 glycoprotein by immunofluorescence and immunoelectron microscopy, 1348

Mutants, temperature-sensitive measles virus, human lymphoblastoid cell lines, 1637

Myasthenia gravis, autoimmune, role of complement in pathogenesis of, 973

Myeloma cell line, mouse, production of IgG$_{2a}$ variants, 1535

Myxoviruses, inborn resistance to, mice, macrophage phenotype, 531

N

Neoplastic cells, cultured, serine enzymes, 745

Neutrophils, human, appearance of Ia-like antigens and complement receptors during maturation of, 730

Nigericin inhibition of mitogen stimulation of human lymphocytes, 571

Nonresponder mice primed with L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}, lymphoid cell immunosuppression, 997

Nuclease, staphylococcal, genetic control of immune response to, gene mapping for anti-nuclease idiotypes, 1517

–, staphylococcal, immune response to, role of non-*H-2*-linked genes in anti-nuclease antibody response, 396

O

Oncornavirus lysis by human serum, identification of complement (C1) receptor as p15E, 844

Ontogeny, B lymphocyte maturation, thymus cell involvement, 196

Orchitis, autoimmune, immunological T-cell memory, autoantigen distribution, 233

P

p15E, complement receptor, oncornavirus lysis by human serum, 844

Pathogenesis of autoimmune myasthenia gravis, role of complement, 973

Peptides, *N*-formylmethionyl, induction of chemiluminescence, phagocytic cells, 182

Phagocytes, mononuclear, culture on Teflon surface to prevent adherence, 271

Phagocytic capacity, membrane receptors, mouse leukocyte differentiation, 434

– cells, chemiluminescence induced by *N*-formylmethionyl peptides, 182

Phenotype, macrophages, mouse, inborn resistance to myxoviruses, 531

Plasma contact activation, surface and fluid phase activities of activated Hageman factor, 719

– membrane, alkaline phosphodiesterase I, mouse peritoneal macrophages, 77

– protein synthesis, embryonic chick hepatocytes, patterns during first three days of culture, 1806

Plasminogen activator, macrophage, regulation by T lymphocytes and specific antigen, Bacille Calmette-Guérin infection, mouse, 1175

– activator secretion, cultured macrophages, glucocorticoid inhibition of, 1695

Platelet function, thrombin-induced, role of complement, 1713

– response to immune complexes and IgG aggregates, effect of lipid A-rich bacterial lipopolysaccharides, 207

Pneumocystis carinii, in vitro interaction with macrophages and L cells, 157

Polyadenylic:polyuridylic acid, effect on generation of cytotoxic T lymphocytes, 1355

Poly-L-(Tyr,Glu)-poly-DL-Ala-poly-L-Lys, immune response to, control by single major histocompatibility complex locus, 599

Postnatal development, mouse, changes in suppressor mechanisms, 87

Potassium ionophore (nigericin), inhibition of mitogen stimulation of human lymphocytes, 571

Procoagulant activity, light chain of human high molecular weight kininogen, 488

Proliferation, anti-Ia-associated, inhibition of, immunoregulatory activity of α-fetoprotein, 667

–, mouse lymphocytes, activation by anti-immunoglobulin, 814

–, T-cell, human erythroid burst-forming unit, 324

Proliferative capacity, loss of in immunohemopoietic stem cells, effects of aging and transplantation, 1526

– capacity, mouse peritoneal macrophages, 1253

– response to *H-2* mutants, T lymphocytes, 1395

– responses, human, to hapten-conjugated cells, 1671

Prostaglandin E production, human blood monocytes, mouse peritoneal macrophages, 952

Prostaglandins, plasma, relationship to ceruloplasmin and haptoglobin, VX_2 carcinoma in rabbits, 1078

Protease deficiency, polymorphonuclear leukocytes, Chédiak-Higashi syndrome, beige mice, 1285

Proteins, connective tissue, fibronectin, distribution in normal human tissues, 1054

– membrane, solubilized, induction of secondary cytolytic T lymphocytes, 946

–, plasma, synthesis of, embryonic chick hepatocytes, patterns during first three days of culture, 1806

–, streptococcal M, nonionic detergent extracted, correlation of immunological cross-reactions and structural similarities, antiphagocytosis, 1771

–, trinitrophenyl-conjugated, induction of *H-2* restricted cytotoxic effectors, 352

Pseudotype, effect on Abelson virus and Kirsten sarcoma virus-induced leukemia, 1044

Pyrogen production, mouse histiocytic and myelomonocytic tumor cell lines, 1503

R

Rabbit, VX_2 carcinoma, relationship of ceruloplasmin and haptoglobin to plasma prostaglandins, 1078

Radiation leukemia virus infection, *H-2* antigens on thymocytes, 470

Rat, bile duct obstruction, secretory IgA and free secretory component in serum, 934

Receptor binding of alloantigens and anti-idiotypic antibodies, activated T cells, 25

Receptors, complement, Ia-like antigens, appearance during human neutrophil maturation, 730

–, complement, p15E, oncornavirus lysis by human serum, 844

–, glucocorticoid, monocytes and macrophages, 1684

–, hapten-binding, idiotypic restriction, T lymphocytes, 1341

–, IgM or IgG, human, T-cell subpopulations, 1405

–, membrane, phagocytic capacity, mouse leukocyte differentiation, 434

–, polymeric immunoglobulins, epithelial cell secretory component, 1832

Responder strains, immunological unresponsiveness to dextran B512, lack of Ig receptor expression, 645

Responsiveness, immunologic, butanol-extracted lipopolysaccharide, C3H/HeJ mouse, 800

Restriction locus, *Bvr-1*, endogenous BALB virus, cat × mouse somatic cell hybrids, 219

Retrovirus expression and autoimmunity, New Zealand black mice, viral envelope glycoprotein gp70, 872

– expression and autoimmunity, New Zealand

black mice, xenotropic virus, 854
Rheumatoid arthritis, induction of nuclear antigen, lymphocyte transformation by Epstein-Barr virus, 1018

S

Schistosoma mansoni, schistosomula, complement-mediated killing of by rat eosinophils, 147
Schistosomula, *Schistosoma mansoni*, complement-mediated killing of by rat eosinophils, 147
Secretory component, epithelial cells, surface receptor for polymeric immunoglobulins, 1832
– IgA and free secretory component, rat serum, bile duct obstruction, 934
Serine enzymes, cultured neoplastic cells, 745
Serotype 2, vaccines, immunogenicity of, specificity of protection against group B meningococcal disease, guinea pig model, 629
Serum, anti-A, insolubilized, *Dolichos biflorus*, competitive binding assays of A_1 and A_2 blood groups, 830
–, human, lysis of oncornavirus, identification of complement (C1) receptor as p15E, 844
–, rat, bile duct obstruction, secretory IgA and free secretory component, 934
– replacement by albumin, transferrin, and soybean lipid, cultures of lipopolysaccharide-reactive B lymphocytes, 923
Sex hormone, effect on survival, anti-nucleic acid antibodies, and glomerulonephritis, NZB/NZW mice, 1568
Skin allografts, murine, effect of anti-H-2K, H-2D, and anti-Ia antisera, 611
– graft rejection, male syngeneic cytotoxic T-cell responses to H-Y, 768
Specificity, restricted, self and non-self *H-2D* antigens, cytotoxic T-cell responses, 1661
Spermatogonia, syngeneic, lysis by cytolytic effector T cells, 251
Spleen-adherent cells, non-B, non-T, immune response gene regulation of accessory cell function of, 1611
–, lymphoblasts, mouse, floatation in bovine albumin solutions, 279
–, lymphoblasts, mouse, in vitro replication and differentiation, 297
Staphylococcal nuclease, genetic control of immune response to, gene mapping for anti-nuclease idiotypes, 1517
– nuclease, immune response to, role of non-*H-2*-linked genes in anti-nuclease antibody response, 396
Stem cell, hematopoietic, regulatory volumes, bg^J/bg^J:W/W^v chimera, 1189
–, cells, immunohemopoietic, loss of proliferative capacity, effects of aging and transplantation, 1526
Streptococcal M protein, nonionic detergent extracted, correlation of immunological cross-reactions and structural similarities, anti-phagocytosis, 1771
Structure, correlation with immunological cross-reactions, streptococcal M protein, anti-phagocytosis, 1771
Suppression, elimination of, in vivo effects of anti-Ia alloantisera, 656
–, mixed lymphocyte reaction, soluble T-cell factor, in man, 137
–, transfer of to normal syngeneic recipients by T or B lymphocytes, 63
Suppressor cell activity, colchicine inactivation of, 1228
– factor, antigen-specific, target and genetics of, 110
– factor, T-cell-, antigen-specific, induction of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} specific suppressor T cells, 123
– mechanisms, changes in during mouse postnatal development, 87
– T-cell induction by lymphoid cells, tolerance to 1-fluoro-2,4-dinitrobenzene contact sensitivity, role of *H-2D* region of major histocompatibility complex, 788
– T cell specific for stimulating alloantigen, mixed lymphocyte reaction, in man, 137
Surface antigen, $G_{(RADA1)}$, mouse leukemia, relationship to murine leukemia virus, 1089
– glycoproteins, murine cytotoxic T lymphocytes, 1418
– immunoglobulin isotype and immune function, murine B lymphocytes, 1374
–, lymphocyte, IgD loss, maturation of memory B cells, 984
– phase activity, activated Hageman factor, contact activation of plasma, 719
– receptor for polymeric immunoglobulins, epithelial cells, 1832
Survival, intracellular, hamster, leishmaniasis, 515
–, NZB/NZW F_1 mice, effect of castration and sex hormones, 1568
Susceptibility, genetic basis, to mouse leukemia induction by 3-methylcholanthrene, 459
– to lysis, target cell, antagonistic inhibition by interferon, 1314
Syngeneic male skin graft rejection, cytotoxic T-cell responses to H-Y, 768
– recipients, transfer of suppression to by T or B lymphocytes, 63
– spermatogonia, lysis by cytolytic effect T cells, 251
– targets at major histocompatibility complex, cell-mediated lympholysis, NZB mice, 1435

T

T cells, activated, binding of alloantigens and anti-idiotypic antibodies to same receptors, 25
– cells, cytotoxic, influenza virus-specific, role of viral infectivity in induction of, 1236
– cells, cytotoxic, recognition of male antigen

and *H-2*, 1291
T cells, cytotoxic, self-specific, plus virus, lymphoreticular system triggering of, role of thymus, 897
– cells, differentiation of *H-2* self-recognition, thymus, 882
– cells, F_1 hybrid, selected to heterologous erythrocytes, helper function, noncollaboration with B cells of opposite parental strain, 1142
– cells, F_1 hybrid, selected to heterologous erythrocytes, helper function, T-B collaboration, mapping to K-end of *H-2* complex, 1159
– cells, helper, Ia$^-$ and Ia$^+$, secondary antibody response, 446
– cells, human, allosensitized, antibody-dependent cellular cytotoxicity, 605
– cells, lymphocyte-activating determinant locus, mapping in *I-C* subregion, 1028
– cells specific for hapten-modified self, precommitment for self major histocompatibility complex antigens, 1065
– cells, suppressor, induction by lymphoid cells, tolerance to 1-fluoro-2,4-dinitrobenzene contact sensitivity, role of *H-2D* region of major histocompatibility complex, 788
T-dependent and T-independent carriers, DNA coupled to, induction of B-memory cells, 1824
T lymphoblasts, mixed leukocyte culture-activated, specific immune unresponsiveness, autoanti-idiotypic killer T cells, suppression transfer to normal syngeneic recipients, 63
– lymphoblasts, mixed leukocyte culture-activated, specific immune unresponsiveness, cellular and serological effects, 50
T lymphocytes, cytolytic, secondary, induction by solubilized membrane proteins, 946
– lymphocytes, cytotoxic, effects of polyadenylic:polyuridylic acid and lipopolysaccharide on generation of, 1355
– lymphocytes, cytotoxic, murine, cell surface glycoproteins, 1418
– lymphocytes, hapten-binding receptors, idiotypic restriction, 1341
– lymphocytes, regulation of macrophage plasminogen activator, Bacille Calmette-Guérin infection, mouse, 1175
– lymphocytes, response to *H-2* mutants, proliferation of, 1395
Target cell susceptibility to lysis, antagonistic inhibition by interferon, 1314
–, syngeneic, major histocompatibility complex, cell-mediated lympholysis against, NZB mice, 1435
T-B collaboration, helper cell induction, F_1 hybrid T cells selected to heterologous erythrocytes, mapping to K-end of *H-2* complex, 1159
T-cell cytotoxic responses to H-Y, Ir gene mapping, 758
–, cytotoxic responses to H-Y, rejection of syngeneic male skin grafts, 768
T-cell cytotoxic responses, self and non-self *H-2D* antigens, restricted specificity, 1661
– factor, soluble, suppression of mixed lymphocyte reaction, in man, 1037
– helper function, role of *H-2*-linked genes, antigen-pulsed macrophages specific for antigen and parental *H-2* products, 554
– helper function, role of *H-2*-linked genes, immune response genes for poly (L-Tyr,Glu)-poly-DL-Ala-poly-L-Lys in B cells and macrophages, 1596
– mediated cytolysis, hexose requirement at lethal hit stage, 1551
– mediated cytotoxic immune responses to F9 teratocarcinoma cells, 251
– mediated lymphocytotoxicity, autologous leukemia-specific, acute myelogenous leukemia, 912
– memory, immunological, autoantigen distribution, autoimmune orchitis, 233
– proliferation in vitro, human erythroid burst-forming unit, 324
– sets, immunoregulatory circuits, feedback inhibition, role of T-helper cells, 1106
– sets, immunoregulatory circuits, role of feedback inhibition, absence in NZB mice, 1116
– subpopulations, human, receptors for IgM or IgG, 1405
– suppression, specific for stimulating alloantigen, mixed lymphocyte reaction, in man, 137
– suppressor factor, antigen-specific, induction of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} specific suppressor T cells, 123
– tumors, mouse, Ly-1, Ly-2, Thy-1, and TL differentiation antigens, 1267
Teflon surface, culture of mononuclear phagocytes on to prevent adherence, 271
Temperature-sensitive measles virus mutants, isolation from infected human lymphoblastoid cell lines, 1637
Teratocarcinoma F9 cells, T-cell-mediated cytotoxic immune responses to, 251
Thrombin, role in uptake of C3 and C5, 1713
Thymocyte precursors, hematopoietic, evidence of heterogeneity, 1843
Thymocytes, *H-2* antigens, radiation leukemia virus infection, 470
Thymus, differentiation of *H-2* self-recognition by T cells, 882
–, lymphoreticular system, triggering of virus plus self-specific cytotoxic T cells, 897
–, role in B-lymphocyte functional maturation, 196
Tissue culture, embryonic chick hepatocytes, patterns of plasma protein synthesis, 1806
– culture, isolated glomeruli, crescentic glomerulonephritis, 98
– culture, mononuclear phagocytes on Teflon surface to prevent adherence, 271
Tissues, normal human, distribution of fibronectin, 1054

Tolerance to 1-fluoro-2,4-dinitrobenzene contact sensitivity, lymphoid cell induction of suppressor T cells, role of *H-2D* region of major histocompatibility complex, 788

Transferrin, albumin, and soybean lipid, replacement of serum, cultures of lipopolysaccharide-reactive B lymphocytes, 923

Transplantation, allogeneic bone marrow, without graft-versus-host disease, total lymphoid irradiation, 963

— immune reactions, anti-idiotypic antibody induction of, 500

—, kidney, long-term *H-2* incompatible, immune status of mice with, 1449

—, kidney, long-term *H-2* incompatible, induced immune destruction, 1469

—, serial, loss of proliferative capacity, immunohemopoietic stem cells, 1526

Trinitrophenyl-conjugated soluble proteins, induction of *H-2* restricted cytotoxic effectors, 352

Tumor, lung, derepressed alloantigen coded by *K* region of *H-2* major histocompatibility complex, 1363

—, mouse histiocytic and myelomonocytic cell lines, in vitro pyrogen production, 1503

Tumor-derived cells, lymphocyte culture, identification of anti-viral activity as interferon, 1299

Tumors, T-cell, mouse, Ly-1, Ly-2, Thy-1, and TL differentiation antigens, 1267

L-Tyrosine^{10}, L-glutamic acid^{60}-L-alanine^{30}-, nonresponder mice primed with, lymphoid cell immunosuppression, 997

—, L-glutamic acid^{60}-L-alanine^{30}-, specific suppressor T cells, induction of, antigen-specific suppressor factor, 123

U

Unresponsiveness, immunological, to dextran B512, lack of Ig receptor expression, 645

—, specific immune, mixed leukocyte culture-activated T lymphoblasts as autoimmunogen, autoanti-idiotypic killer T cells, suppression transfer to normal syngeneic recipients, 63

—, specific immune, mixed leukocyte culture-activated T lymphoblasts as autoimmunogen, cellular and serological effects, 50

V

V genes, nonrandom expression, immunological unresponsiveness to dextran B512, 645

Vaccines, serotype 2, immunogenicity of, specificity of protection against group B meningococcal disease, guinea pig model, 629

Virus, Abelson, induction of leukemia, effect of pseudotype, 1044

—, Abelson, induction of lymphoid cell transformation, effect of helper virus, 1126

—, endogenous BALB, cat × mouse somatic cell hybrids, *Bvr-1* restriction locus, 219

—, envelope glycoprotein gp70, autoimmunity and retrovirus expression, New Zealand black mice, 872

—, Epstein-Barr, lymphocyte transformation, induction of nuclear antigen, rheumatoid arthritis, 1018

—, Friend leukemia, genetic resistance to, gene (*Fv-3*) regulating immunosuppression, 422

—, Friend, particles, nonrandom inclusion of *H-2K* and *H-2D* antigens, 340

—, helper, effect on Abelson virus-induced transformation of lymphoid cells, 1126

— infection, radiation leukemia, *H-2* antigens on thymocytes, 470

—, influenza, cytotoxic T cells, role of viral infectivity in induction of, 1236

—, measles, temperature-sensitive mutants, isolation from infected human lymphoblastoid cell lines, 1637

—, murine leukemia, relationship to cell surface antigen $G_{(RADA1)}$, mouse leukemia, 1089

—, myxo-, inborn resistance to, mice, macrophage phenotype, 531

— plus self-specific cytotoxic T cells, lymphoreticular system triggering of, role of thymus, 897

—, xenotropic, autoimmunity and retrovirus expression, New Zealand black mice, 854

Virus-transformed cells, lymphocyte culture, identification of anti-viral activity as interferon, 1299

Volumes, regulatory, hematopoietic stem cell, bg^J/bg^J:W/W^v chimera, 1189

VX$_2$ carcinoma, rabbits, relationship to ceruloplasmin and haptoglobin of plasma prostaglandins, 1078

X

Xenotropic virus, autoimmunity and retrovirus expression, New Zealand black mice, 854

X-linked, hapten-specific B-cell blockade, CBA/N mice, 1755

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1854 Index to Volume 147

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CONTENTS THE JOURNAL OF EXPERIMENTAL MEDICINE

Volume 147 No. 6 June 1, 1978

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1551 Ian C. M. MacLennan and Pierre Golstein. Requirement for hexose, unrelated to energy provision, in T-cell-mediated cytolysis at the lethal hit stage

1568 J. R. Roubinian, N. Talal, J. S. Greenspan, J. R. Goodman, and P. K. Siiteri. Effect of castration and sex hormone treatment on survival, anti-nucleic acid antibodies, and glomerulonephritis in NZB/NZW F_1 mice

1584 Eva Engvall, Erkki Ruoslahti, and Edward J. Miller. Affinity of fibronectin to collagens of different genetic types and to fibrinogen

1596 Philippa Marrack, and John W. Kappler. The role of H-2-linked genes in helper T-cell function. III. Expression of immune response genes for trinitrophenyl conjugates of poly-L(Tyr, Glu)-poly-D,L-Ala-poly-L-Lys in B cells and macrophages

1611 Alfred Singer, Carol Cowing, Karen S. Hathcock, Howard B. Dickler, and Richard J. Hodes. Cellular and genetic control of antibody responses in vitro. III. Immune response gene regulation of accessory cell function

1621 Mikael Jondal and Stephan Targan. In vitro induction of cytotoxic effector cells with spontaneous killer cell specificity

1637 Grace Ju, Stephen Udem, Bracha Rager-Zisman, and Barry R. Bloom. Isolation of a heterogeneous population of temperature-sensitive mutants of measles virus from persistently infected human lymphoblastoid cell lines

1653 J. G. Knight and D. D. Adams. Three genes for lupus nephritis in NZB x NZW mice

1661 Robert V. Blanden and Ursula Kees. Cytotoxic T-cell responses show more restricted specificity for self than for non-self *H-2D*-coded antigens

1671 Michael F. Seldin and Robert R. Rich. Human immune responses to hapten-conjugated cells. I. Primary and secondary proliferative responses in vitro

1684 Zena Werb, Rosemary Foley, and Allan Munck. Interaction of glucocorticoids with macrophages. Identification of glucocorticoid receptors in monocytes and macrophages

1695 Zena Werb. Biochemical actions of glucocorticoids on macrophages in culture. Specific inhibition of elastase, collagenase, and plasminogen activator secretion and effects on other metabolic functions

1713 Margaret J. Polley and Ralph Nachman. The human complement system in thrombin-mediated platelet function

1727 Margrit P. Scheid, Gideon Goldstein, and Edward A. Boyse. The generation and regulation of lymphocyte populations. Evidence from differentiative induction systems in vitro

1744 Jan Andersson, Antonio Coutinho, and Fritz Melchers. The switch from IgM to IgG secretion in single mitogen-stimulated B-cell clones

1755 B. Merchant, H. Snippe, E. F. Lizzio, and J. K. Inman. Cellular and molecular requirements for X-linked, hapten-specific B-cell blockade in CBA/N mice

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1779 Eric A. Jaffe and Deane F. Mosher. Synthesis of fibronectin by cultured human endothelial cells

1792 Daniel H. Conrad, Jaime R. Carlo, and Shaun Ruddy. Interaction of $\beta 1H$ globulin with cell-bound C3b. Quantitative analysis of binding and influence of alternative pathway components on binding

1806 Gerd Grieninger and S. Granick. Synthesis and secretion of plasma proteins by embryonic chick hepatocytes. Changing patterns during the first three days of culture

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