ANNUAL REPORT
OF
PROGRAM ACTIVITIES
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
FISCAL YEAR 1979

U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
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NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

October 1, 1978 Through September 30, 1979

Nineteen Seventy-Nine has been a busy year for the NIAID. This is reflected in the reports of the program directors and branches and laboratories which follow. We have cause for pride in the accomplishments of our scientific constituency and the momentum that is being built toward solution to many of the problems that have beset us and been insolvable.

The Director has devoted much of his time to interaction with constituent groups. These efforts to inform them of the Institute's research programs, learn their views and enlist their interest and support have opened new pathways to information about the problems of the practicing physician and his views on priorities. In January 1979, the Institute held an open house which was attended by representatives of 24 professional societies and voluntary health organizations. This all-day meeting was devoted to exchange of information and discussions. The Director also gave the following invited lectures during the year.

Diabetes, Virology and DNA or It's a Long Way from Amphioxus. October 1, 1978, Annual ICAAC Lecture, Atlanta, Georgia.

Introductory Remarks. NIAID 30th Anniversary, October 27, 1978, Masur Auditorium, NIH, Bethesda, Maryland.

The Influence of Infection of the Geography of Heart Disease. November 13-15, 1978, T. Duckett Jones Memorial Lecture, 51st Annual Meeting of the American Heart Association, Dallas, Texas.

The Second Century of Medical Bacteriology: Rediscovered Opportunities. November 20, 1978, American Society for Microbiology Archives Collection, Catonsville, Maryland.

From VD to STD (Sexually Transmitted Diseases). November 21, 1978, Medicine for the Layman, Masur Auditorium, NIH, Bethesda, Maryland.

A Tribute to Miss Helen Hayes. November 28, 1978, Asthma and Allergy Foundation of America, New York, New York.

Conference on Pharmaceuticals for Developing Countries. January 21-31, 1979, National Academy of Science, Washington, D.C.

A Return to Nature: A Challenge for Outdoor Medicine. February 2, 1979, Columbia Medical Society, Columbia, South Carolina.

International Symposium on Potentiation of Immune Response to Vaccines. February 20-21, 1979, Wilson Hall, NIH, Bethesda, Maryland.

From Glendalough to the Great Famine: The Unexpected in Human History. March 30, 1979, Annual Meeting of the American Epidemiology Society, Atlanta Hilton Hotel, Atlanta, Georgia.

Can Curiosity Flourish in an Age of Diminishing Expectations? April 5, 1979, Rosenstiel Basic Medical Sciences Research Center, Waltham, Massachusetts.

The New York Academy of Medicine Medal for 1979 to Dr. Maclyn McCarty. April 19, 1979, The New York Academy of Medicine, New York, New York.

INTRODUCTORY REMARKS: The Kinyoun Lecture. "The Role of Complement in Natural Resistance to Infections." April 24, 1979, Wilson Hall, NIH, Bethesda, Maryland.

A Claim for Curiosity on a Vanishing Frontier. May 4, 1979, Center for Interdisciplinary Research in Immunologic Diseases (CIRID), University of California, Los Angeles, California.

INTRODUCTORY REMARKS: The Kinyoun Lecture. "Cell Mediated Immunity to Intracellular Parasites and Polymorphic Nature Transplantation Antigens." May 8, 1979, Wilson Hall, NIH, Bethesda, Maryland.

Cystic Fibrosis and the Historical Precedent for Optimism. August 9, 1979, The Cystic Fibrosis Foundation President's Conference, Dulles Airport, Virginia.

"Role of the National Institute of Allergy and Infectious Diseases in the Conquest of Disease -- A Tenacious Strategy to Match a Restless Tide." September 21, 1979, Wilson Awards Symposium: Prevention of Major Infectious Diseases: Current Concerns and Future Promise, Rochester, New York.

Dr. Jack S. Whitescarver has attended a number of professional society and voluntary health organization meetings to meet with officers and others regarding relationships between them and the Institute. He is assuming a major role in the development of fiscal and program information desired by these organizations, working closely with the Director and Program Directors in doing so.

International Medical Research

The absence of a full-time professional with responsibility for international medical research activities threw quite a burden on the Director and Deputy Director. A new initiative in the government to develop research and technological exchanges and activities with the People's Republic of China (PRC) required development of suggested program activities. With the signing of a formal agreement between the Ministry of Health, PRC, and the DHEW, the NIAID drew responsibilities for collaboration in infectious diseases, immunology

and recombinant DNA research. The activities now involving program staff have required representation at a number of meetings. As the year ends, definitive activities are evolving.

The Director has also been heavily involved with the Office of International Health in reviewing possibilities for expanded relationships with medical research in India. This stems from requests by the Indian Medical Research Council and involved a trip to India for discussions.

The Deputy Director was designated as Chairman of the Subcommittees on Biomedical Research and Infectious and Parasitic Diseases of the U.S.-Egypt Committee on Medical Research. In this role he attended the joint meetings of the subcommittees and committees in July where the entire collaboration in research was reviewed, pending applications reviewed and approved, and plans made for the allocation of the rapidly dwindling residual of PL 480 funds.

Also as the year ended, new initiatives in cooperation with the Japanese were developing. These stemmed from interests of Frank Press, Director of the Office of Science and Technology Policy, The White House, in increasing the investment being made by the Japanese in research and technology which potentially benefits the U.S. A July, 1979 trip in which the Director, NIH, participated led to agreement in several areas involving the NIAID. These were vaccine development, immunology, and recombinant DNA research. The Director is working with the Director, NIH, and staff toward specific proposals in these areas. Immunology will eventually wind up within the framework of the U.S.-Japan Cooperative Medical Sciences Program administered by the NIAID. Vaccine development may also go this route, but recombinant DNA will require new mechanisms for cooperation.

Influenza

The Deputy Director, along with the Director, MIDP, serves on an Interagency Working Group (IWG) for DHEW activities in the control of influenza.

The IWG has coordinated responses to a number of questions regarding influenza stemming from the Secretary's Conference in 1978, and the Congress and the OSTP. It has also maintained an exchange of information between the CDC, the NIAID, and the BoB/FDA on occurrence of the disease, virus strain characteristics and research activities. Other activities, including the Consensus Conference on Amantadine, are described in the MIDP report.

Recombinant DNA Activities

The Office of Specialized Research and Facilities continued to maintain high containment facilities in an operational state throughout FY 79. The laboratory at the Frederick Cancer Research Center provided the main facility support for risk assessment experiments being conducted by Drs. Rowe and Martin of the Intramural Program. Those experiments have now provided the first direct evidence that some potential biohazards considered earlier are unlikely to occur. It is anticipated that those results, and others yet to come from continuing studies, will have a major impact on revisions of the

NIH Guidelines for Recombinant DNA Research. The 1978 Guidelines do not explicitly require P-4 containment for any permissable experiment. Consequently the laboratory in Frederick is being operated at lower levels but it can convert to P-4 operations on very short notice should a need arise. The Mobile Containment Laboratory has been downgraded to a P-3 facility and will not be operated again in the P-4 mode.

During the year, the Architectural/Engineering studies for the National Biomedical Containment Laboratory were completed in compliance with the original workscope. The A/E construction cost estimates were significantly higher than those developed by both staff and the prime contractor, Litton Bionetics, Inc., at the beginning of the project. Review of the A/E estimates revealed them to be accurate and reasonable but significantly higher than estimating data references for general research laboratories; this reflects the extremely complex nature of the NBCL. Accordingly, the A/E has been asked to restructure the drawings to permit a basic bid package with "add-ons" to accomplish the construction of the entire building in stages. Receipt of the revised drawings is anticipated near the end of FY 79 and a decision as to whether to solicit construction bids is expected at that time.

With the issuance in December 1978 of revised guidelines, the Secretary, DHEW, requested that the NIH prepare a Risk Assessment Plan. Since the hypothetical risks and technical basis of recombinant DNA research are primarily microbiological in nature, the responsibility for coordination and implementation of the plan was assigned to the Director, NIAID. In compliance with the Secretary's request, the NIAID published a proposed plan for public comment and had that document reviewed by the Recombinant DNA Advisory Committee. This first Annual Plan has now been published in the Federal Register. In implementing the Plan the NIAID will: (1) recruit and appoint an eminent scientist as a Special Assistant to the Director for Risk Assessment to provide leadership and coordination of all activities concerned with the evaluation of risks; (2) develop and issue such requests for applications or proposals as are necessary to ensure the conduct of risk assessment research required to answer specific questions or to fill gaps in data being accumulated from other research; (3) prepare and send periodic reports to the RAC identifying questions, problems, and evaluations of scientific information pertinent to their various advisory functions; and (4) respond to inquiries from scientists, the public, DHEW, or other government agencies regarding available data on risk assessment and evaluation of those data. In carrying out these responsibilities, the NIAID will enlist the services of existing NIH offices, committees, and people to provide information, to advise and evaluate, and to review, as appropriate, reports for completeness and accuracy.

The consolidation by NIH of recombinant DNA administrative activities into one Institute was continued by the decision to transfer the Office of Recombinant DNA Activities from NIGMS to NIAID. As FY 79 draws to a close, the operational aspects of the transfer have all been effected and it is anticipated that at the start of FY 80 final administrative approval will be received.

Office of Research Reporting and Public Response

The loss of experienced professional and secretarial staff during this year weakened the efforts of ORRPR. Assistant Chief Margaret McElwain had been a member of our staff for more than 10 years; Ms. Bobbie Plocinik, our "resident immunologist," had been with us for more than five years, and Ms. Sylvia Glukenhaus had been ORRPR secretary for more than eight years. These personnel losses hitting ORRPR in one year resulted in a reduction in overall productivity, particularly in the area of research reports.

We were successful in recruiting Mr. Thomas Coleman, former executive director of the National Association of Hearing and Speech Agencies, to serve as an expert in medical communications. He will be developing the Institute's capability in the audiovisual and electronic media. He also will be available to work with the Director and other Institute personnel on communications projects, including "outreach" programs and minority efforts. Also, Ms. Lydia Woods Schindler has been employed under contract to work on a variety of writing assignments with Dr. Krause.

Publications -- Much of ORRPR's staff effort this year went into producing publications which were a part of or an outgrowth of two NIAID task forces. As a result, NIAID now has the beginnings of a Scientific Monograph Series with a six-volume report of the Virology Task Force and a single volume reporting the Task Force on Asthma and the Other Allergic Diseases. Our staff devoted a great deal of time in editing, proffing and distributing these monographs to appropriate scientists and physicians. In addition, we helped to prepare and distribute summary reports of the Virology Task Force chairman and the Asthma and Allergic Disease Task Force. The latter summary report contained NIAID Council Recommendations.

One aspect of both Task Force charges was to produce a document aimed at the general public. The publication, Allergies and Asthma--An Optimistic Future, was written under contract by Patrick Young, freelance science writer. Approximately 70 illustrations were selected and developed by ORRPR staff to be included in this lay version, which is scheduled for distribution early next year. The manuscript for the lay publication in virology, written under contract by Elaine Blume Wilson, a former ORRPR staffer, has the working title of Intimate Enemies--An Introduction to Viruses. It also is in final stages of preparation and is being considered as an "occasional paper" from the Institute.

Our staff assisted in the preparation of the Institute's chapter on "Tobacco --Its Role in Allergy and Immunity," for the DHEW publication, Smoking and Health: A Report of the Surgeon General. Copies of this chapter have been reprinted and are being distributed to the nation's allergists.

ORRPR has collaborated with NIH's Medical Arts Department in the design of a new format for our fact sheet series. The new format is half the former page size, easier to display and store, more attractive and economical. Six fact sheets have been printed in the new format.

Other publication efforts include a new fact sheet on hereditary angioedema, a backgrounder on staph infections, and a Q and A on genital herpes; major revisions in three pamphlets -- "Asthma," "Sexually Transmitted Diseases," and "Drug Allergy" -- and revisions of four fact sheets -- "Malaria," "Toxoplasmosis," "Viral Hepatitis," and "Rocky Mountain Spotted Fever."

We continue to prepare columns for the "Search for Health" series distributed by the NIH to local newspapers around the country. Over the years, the response to these health columns has been excellent with thousands of follow-up requests for NIAID publications. This year's columns resulted in requests for our publications on pollen allergy, insect allergy, poison ivy allergy, and Rocky Mountain spotted fever.

We also have made a special effort to supply allergy publications on a continuing basis to the local chapter of the Asthma and Allergy Foundation of America for distribution at its public meetings, which are often attended by an ORRPR staff member.

In our continuing cooperation with the Consumer Service (CIS), ORRPR provided copies of the backgrounders on "Acne" and "Q&A About Allergies," both of which were promoted in the spring and summer CIS catalogues. A CIS spokesperson has advised ORRPR that in response to requests approximately 90,000 copies of "Acne" and 75,000 copies of "Q&A About Allergies" have been distributed this year.

In total, more than a quarter of a million NIAID publications were distributed by ORRPR during the year.

Public Inquiries -- The number of public inquiry requests continues to increase. Approximately 700 individualized letters were prepared to respond to a variety of requests, including nearly 40 responses to the Congress.

Scientific Meetings and Information -- During the past year, ORRPR staff managed press briefings tied to the International Symposium on Pertussis, the International Symposium on Potentiation of Immune Response to Vaccines, and the results from the initial polyoma recombinant DNA risk assessment experiments conducted by NIAID scientists (Martin and Rowe) at Ft. Detrick.

Working closely with Institute scientists, representatives of NIH's Office of Medical Applications for Research (OMAR) and a private contractor, ORRPR staff developed promotional materials and advance publicity for the NIH Consensus Development Conference on the Use of Amantadine. This conference, to be held on October 15, 1979, will be followed by a press briefing and the recommendations of the conference panel will be disseminated by ORRPR staff.

Exhibits were produced and manned at the annual meetings of the following organizations: American Public Health Association (APHA): the Association of Military Surgeons of the U.S.; the American Academy of Allergy; the American Society for Microbiology (AMS); and the American Lung Association/ American Thoracic Society. This represents an increased effort in reaching

members of NIAID "constituent" organizations. ORRPR staff also assisted NIAID's EEO Coordinator in developing an exhibit for the Minority Biomedical Science Symposium in Atlanta. We furnished pamphlets and information on infectious diseases for two panels of an NIH Year-of-the-Child exhibit, which is being sent to many meetings, including next year's APHA meeting.

At the ASM meeting, ORRPR employed a videotape presentation for the first time. This included two short segments: (1) a description of NIAID's high-risk containment facility at Ft. Detrick; and (2) scientific footage, provided by Dr. Adel Mahmoud of Case-Western Reserve University, visualizing an antibody potentiated attack on trichina larvae by eosinophils.

Last year, ORRPR staff developed our first patient-education slide/tape show on "What You Should Know About Asthma." Because of its success, we were developing a second show, "Coping with Your Allergy -- At Home, At School and On the Job." This was previewed at our exhibit at the American Academy of Allergy meeting. It will be pretested in selected patient populations next year for suitability of material and its comprehension level prior to being offered to the General Services Administration (GSA) for possible sale. GSA has reported that nearly 300 copies of "What You Should Know About Asthma" have already been sold to physicians, hospitals, and nonprofit organizations. In addition, ORRPR has lent copies of the show to several groups, including the PTA, Bronco Junction (a camp for asthmatics) and local chapters of the Asthma and Allergy Foundations of America and the American Lung Association.

Special Events -- ORRPR handled publicity in connection with the Institute's 30th anniversary celebration. Members of the Institute staff attending the formal ceremony in Masur Auditorium on October 27 received a special four-page feature of NIAID, prepared by ORRPR staff, which later appeared in the NIH Record's special anniversary issue.

ORRPR worked closely with Dr. Krause and Dr. Gordon Wallace on the program, poster and plaque design for the NIAID Kinyoun Lecture Series. Established by Dr. Krause (with Dr. Fredrickson's verbal approval), this new series of invited lecturers will, for the most part, emphasize the interdependence of infection and immunity. The first two lecturers were Dr. Hans Muller-Eberhard and Dr. Rolf Zinkernagel, both from Scripps Clinic, La Jolla, California.

EEO Activities and Functions

Report from the Chairperson, EEO Advisory Committee -- The EEO Advisory Committee consists of 16 voting members. Twelve were elected by the NIAID staff, two were appointed by Dr. Krause, and two are the NIAID EEO Counselors for the Bethesda campus. There is an additional EEO Counselor at the Rocky Mountain Laboratory and she participates in a newly organized EEO Advisory Subcommittee for RML. In addition to active participation by the RML Subcommittee, a Federal Women's Program Subcommittee has some members on the Advisory Committee and some other Institute members. The Federal Women's Program Coordinator (FWPC) for NIAID heads the FWP

Subcommittee and is an ex officio member of the Advisory Committee. Also participating as ex officio members are the Training Specialist and Coordinator for Employment of Handicapped and the Hispanic Employment Program Coordinator, along with the EEO Coordinator for NIAID. Members of the Office of the Director, Personnel Office, Information Office, Scientific Director's Office and others in various divisions of management participate in advisory capacities. The EEO Advisory Committee is directly advisory to the Director on all matters relating to EEO concerns, programs and objectives. Much of the committee's activities involve monitoring of the existing Affirmative Action Plan, encouraging completion of EEO program objectives and development of new EEO and affirmative action objectives. A summary of recent work is presented in this report.

Reports from Subcommittees of the EEO Advisory Committee -- Chairpersons for various subcommittees of the EEO Advisory Committee prepared final reports on activities conducted by their subcommittees during the past fiscal year. These detailed reports, which were submitted to the Chairperson of the EEO Advisory Committee, are summarized below:

1. Subcommittee on Program Evaluation -- The subcommittee recognized the need for a system to monitor the implementation of the Affirmative Action Plan (AAP) for the NIAID. It recommended that the Major Initiative Tracking System (MITS), or a similar system, be used by the EEO Advisory Committee to facilitate program evaluation in this regard. This tracking system entails (a) the selection of the most impacting objectives from the AAP for tracking, (b) the collection of monthly progress reports from responsible officials, (c) the charting of progress made with respect to the attainment of these objectives on a quarterly and annual basis, and (d) the dissemination of all charted information to NIAID employees at the end of each fiscal year. By means of this tracking system, the EEO Advisory Committee should be made aware of problems and/or obstacles that may impede progress in implementing the AAP. The subcommittee acknowledged that the EEO Coordinator is involved in monitoring the implementation of the AAP. However, the EEO Advisory Committee, as an elected body, has a special obligation to carefully and systematically monitor affirmative action programs. This may be done in conjunction with the EEO Coordinator's office, but it should also be done by the EEO Advisory Committee separately.

2. Subcommittee on Statistics -- This subcommittee is responsible for collecting, interpreting, and reporting statistical information to the EEO Advisory Committee for further evaluation. In order to perform these functions, access to a vast source of statistical information must be obtained. Since this type of information is not presently available to the subcommittee, it is exploring the possibility of establishing a computerized personnel master file for NIAID. This file would be accessible to both the EEO Advisory Committee and the EEO Coordinator. The purpose of such a file would be to provide up-to-date information on current trends in hiring, promotions and transfers; it would serve as a means for detecting flagrant deficiencies in these areas.

3. Subcommittee on Training -- On the basis of the response to a questionnaire submitted to employees of the NIAID, the subcommittee prepared an extensive report on training opportunities within the NIAID. Several barriers to training were identified and suggestions were made as to how these barriers might be overcome. The subcommittee's general recommendations included (a) the proper distribution of NIAID policy statements on training opportunities to all interested employees, (b) increased efforts to eliminate advancement barriers for research employees, (c) the requirement that all new employees be informed of NIAID-sponsored training programs, (d) the encouragement of supervisors to discuss training possibilities with employees, (e) communication on possible continued employment to stay-in-school employees now on board, and (f) a request for training slots for employees in the animal care and secretarial job series.

4. Subcommittee on Communication -- The major function of this subcommittee is to disseminate information on EEO activities to employees within the NIAID and to seek ways by which this communications process and awareness of the objectives of EEO programs can be improved. The subcommittee is currently seeking input from employees within the NIAID on the format, agenda, and organization of the coming EEO Workshop at which a new five-year AAP will be formulated.

5. Subcommittee on Awards -- This ad hoc subcommittee reviewed several nominations for annual EEO awards. In accordance with NIH guidelines for service and dedication to the principles of EEO, three nominees were selected for awards and special recognition: Ms. Thelma Gaither, LCI; Dr. Richard Asofsky, LMI; and Ms. Edna Miller, OD. These individuals were presented with certificates and cash awards, in recognition of their outstanding contributions to the EEO program, at the NIAID All Hands Staff Meeting on June 21, 1979.

6. Report from the Coordinator of the Federal Women's Program -- Late in 1977, nominees or volunteers for a Federal Women's Program Coordinator (FWPC) were requested of the NIAID staff. Selection was made by the EEO Coordinator (Vincent Thomas) with Advisory Committee concurrence. Weltha Logan (Bio Lab Tech, LMI) was Dr. Krause's appointment for a two-year collateral duty, from September 1977 to 1979.

   A brief survey was conducted by Ms. Logan, in the course of a month's detail in January 1978, to assess the concerns of the women of the Institute and to determine support for an NIAID Federal Women's Program. Major among the concerns listed by the responders were those of position management, career advancement, and training.

   A subcommittee of the Advisory Committee was the preferred organizational choice of management, based on the desire to maintain all EEO concerns as an entity. Ten members, selected from among responders to the survey and representing various Institute programs and EEO purviews, were appointed in September 1978. They received training in November 1978 to assist them in understanding their functions and to set

specific goals for the coming year. Working groups were established for (a) survey, (b) program, (c) communications, and (d) network. Also, an ad hoc group was established to formulate an amendment to the Advisory Committee charter describing the subcommittee.

Prior to the establishment of the subcommittee, group discussion lunches had been scheduled periodically. Early in 1978, their purpose was to discuss possible goals for the program. Later, Ms. Rachelle Mandlebaum of the Employee Assistance Branch spoke on "Coping with Stress on the Job." Dr. Elizabeth Hearne of NIGMS discussed statistics on women in the sciences; and Alice Sargent, an organizational consultant, spoke on women in management.

Presently the subcommittee is working mainly in two areas: program and survey. Programs approved and scheduled include: (a) two presentations for Secretaries' Week on effective communications by Dr. Gloria Harris, offered to all of NIAID's office support staff; and (b) a two-hour seminar open to all of NIH, entitled "Women in the Workforce." Dr. Ann Briscoe, biochemist at Harlem Hospital Center in New York City, spoke on the problems faced by professional scientific women. To speak on issues which affect all women who work was Dr. Fierst, economist and member of the Interdepartmental Task Force on Women established by the White House.

Survey questions have been formulated and sent around for review; these suggestions were revised for final approval by the EEO Advisory Committee and EEO Team. It is hoped that information received from the survey will initiate action items for the five-year AAP to be devised this summer.

Meetings of the Federal Women's Subcommittee are scheduled for the third Wednesday of each month from 12:00 to 1:30, held alternately in the Westwood Building and Building 31. The meetings are open to visitors. Minutes of meetings are distributed to the Institute with the minutes of the NIAID EEO Advisory Committee meetings and posted for staff perusal.

Special Projects Related to the Affirmative Action Plan.

1. Recruitment Initiatives

   A. Outreach to Local Minority High Schools -- A program was developed during the past fiscal year to stimulate minority and women's interest in biomedical careers. Students from four high schools in the District of Columbia (Wilson, McKinley, Coolidge, and Spingarn) were invited to visit the NIAID; students from more high schools could have been invited, but the four-week D.C. teachers' strike created serious communication problems in this regard. Each visiting group was comprised of from 12-17 students plus at least one of their teachers. The program for the students consisted of a welcome and orientation session at which the Scientific Director presented background information on research programs being conducted at NIH

and NIAID; also, there was a discussion of volunteer and salaried positions available for high school students within the NIAID. Then students were escorted to several laboratories where members of the scientific staff explained the type of research being conducted. Here, the students had an opportunity to observe experiments actually in progress and the operation of instruments and/or techniques used in biomedical research. Although Dr. Lois A. Salzman, LBV, was largely responsible for planning and coordinating all aspects connected with this program, the individual efforts of many senior staff scientists contributed greatly to its success.

B. Introduction to Biomedical Research -- The NIAID recently hosted 39 outstanding science students who participated in a seminar, Introduction to Biomedical Research. The students who came from 20 states, the District of Columbia, and Puerto Rico were chosen by their colleges to participate in this special program held February 27 to March 1. The seminar was part of the Minority Biomedical Sciences Program that assists financially and educationally disadvantaged students to enter biomedical research and health-related professions.

Dr. Zora J. Griffo, OD, and Dr. Richard M. Krause, Director of NIAID, welcomed the students and discussed career opportunities at NIH. Dr. Ciriaco Q. Gonzalez, Chief, MBS Program, DRR, emphasized the need for minority participation in the biomedical research sciences. The students also heard from NIAID intramural scientists and Dr. Kenneth Sell, the Institute's Scientific Director.

The students visited NIAID laboratories where they spoke with investigators and observed various experiments in progress. They were also interviewed by researchers for their interest in NIH's summer employment program; 10 of these students were invited to return to NIAID in the summer to work in various laboratories.

The program was organized by Dr. Kathleen Jaouni Cook, assistant to Dr. Sell, and Mr. Frank Fountain, NIAID's EEO Coordinator.

2. Community Affairs

The Community Outreach Program -- As a follow-up to the Personnel Office's participation in a Career Fair held at St. Paul's Christian Community Church, 414 Tennessee Avenue, N.E., in June of 1978, the following activities were initiated and are now in progress: a tutorial reading service; an alcohol and drug abuse program; and safety and security workshops.

3. Employee Development Programs -- The Employee Development Specialist (Mrs. Edna Miller) instructed and coordinated a course in Laboratory Animal Care for 16 employees, as well as coordinated three seminars on the Factor Evaluation System. The latter were designed for administrative and supervisory personnel. Mrs. Miller has established a Mini

Information Center for all employees in her office (Room B1-01, Building 5). Catalogs and a variety of information on educational opportunities for various career specialties are available. In addition to these activities, tours of the Institute were conducted for summer employees, new employees, and administrative personnel; such tours are scheduled to be held at least four times each year.

4. Employment of the Handicapped -- The Coordinator for the Handicapped Persons Program (Mrs. Edna Miller) reported that three handicapped employees successfully completed a course in Laboratory Animal Care in April 1979. The Coordinator has attended two courses so as to keep abreast of policies and laws concerning (a) the selective placement of the handicapped, and (b) cooperative training and employment programs for the handicapped.

Report from the Office of the EEO Coordinator -- The National Institute of Allergy and Infectious Diseases is undertaking a program of affirmative action to which good faith efforts will be directed for achievement of its Major Civil Rights and Affirmative Action Initiatives. Our goals and initiatives are consistent with those of DHEW, PHS, and NIH. In addition, goals and initiatives have been established which are unique to NIAID.

The EEO Coordinator has direct access to the Institute Director. Additionally, the EEO staff has direct access to all NIAID personnel, including the Executive Officer, Personnel Officer, and Associate Directors. There are Federal Women's Program and Hispanic Employment Program Coordinators assigned on a part-time basis, each spending at least 20 percent of their time toward accomplishing program objectives. It has been difficult for both the FWPC and HEPC to actively pursue program objectives based on a 20 percent time allocation. All other EEO officials who have other principal assignments devote at least 20 percent of official time to EEO program matters and serve as technical advisors to the Personnel Officer. The Institute has an EEO Advisory Committee and three EEO Counselors. Adequate management and fiscal controls are established to track all resources devoted to the EEO program and expenses have been charged to, and tracked by, the EEO Coordinator and Budget Office.

Our present recruitment sources do yield qualified minority and female applicants who meet organization needs; however, there has not been an adequate number of minorities to select from in the M.D. and Ph.D. community. Our recruitment literature reflects the desire to reach all segments of the potential for employment, and this effort is also across internal NIH organizational lines to obtain maximum effectiveness and efficiency. All paid advertisement used for recruitment includes minority media coverage. These recruitment efforts are monitored to ensure equal treatment regardless of race, color, religion, sex, national origin, handicap, and age.

Plans are being developed to increase the representation of Hispanics and Native Americans through our Outreach Programs.

NIAID's Extramural Activities Program, through training grants and contracts, participates in the Minority Access to Research Careers Program (MARC) which is designed to help minority institutions train greater numbers of scientists and teachers in the biomedical discipline. Presently, two postdoctoral fellows are being supported through this program. No new hires resulted from this program during FY 1979.

Minority Biomedical Support Program (MBS) is designed to increase minority participation in biomedical research, to develop a pool of minority scientists, to strengthen biomedical research capabilities of minority institutions, and to utilize the talents of minority biomedical investigators in the mission of the NIH. NIAID contributed $125,858 during FY 1979 as part of its support agreement.

NIAID is supportive of the Cooperative Education Program and has two Black female biomedical student participants.

Expert appointments are those appointments of individual scientists or professionals qualified in fields related to the program needs of NIAID. Two white females have received appointments.

Intergovernment Personnel Agreement (IPA) -- One Black female is presently in our Laboratory of Clinical Investigation.

The Clinical Associates Program, class of 1979, provided two white females.

NIAID is committed to increasing the participation of minorities and women in the scientific community and in those grades and occupational series where there is underrepresentation. No hiring goals were set for any of our recruitment programs during FY 1979.

Sixty employees responded to a qualifications analysis questionnaire. The survey was forwarded to all nonprofessional employees. A consultant was hired for this project. During the follow-up, approximately 45 NIAID employees were interested in a qualifications analysis. Workshops were conducted which focused on specifically identified career fields, i.e., personnel, administration, program analysis, etc. Employees were counseled as to their specific qualifications, what was needed for entry into primary career fields of their interest, and exposed to the prospective opportunities at NIH in those fields. Two such surveys were conducted during FY 1979, one by NIH. The skills file is referred to when considering applicants for all vacancies.

Three workshops were conducted on the Factor Evaluation System. The content of these workshops was designed and geared to the needs/interests of the audience. A film was shown and a discussion was held on how this system would affect those individuals in the auditing and classification of their positions.

Training opportunities are available for all employees to facilitate their career goals. All denials of training must be supported in writing. An

analysis of training sponsored by the Institute is ongoing to determine if there is any maldistribution by sex, race, grade, etc.

The majority of this Institute's supervisors and managers have received supervisory and EEO training. An assessment is being conducted to examine our supervisory and management practices and to identify our needs for implementing training in specific areas of concern. The Institute is committed to the assurance that all managers and supervisors will receive training in accordance with established principles.

This Institute supports the NIH Upward Mobility Programs including the Merit Promotion plan. At present we have no internal training programs or positions. However, NIAID provides training for other Institutes' Management Interns. Career counseling is available to all employees through our Employment Development Specialist.

The EEO Plan is updated annually. The various program evaluations are incorporated into the plan as changes or additions at that time. The evaluation is conducted by the EEO Coordinator, the Director, the EEO Advisory Committee, and the EEO Team. Other management officials participate when the topics to be discussed require their input. An AAP Update Workshop is planned for the transition year. One of the objectives is long- and short-range AAP objectives. The EEO Advisory Committee will receive EEO Training for Advisory Committee Members. This will also facilitate monitoring and evaluation of our EEO Program.

Currently, there is no program in effect to cross-train the EEO Specialists or the Personnel Management Specialists. The Personnel Management Specialists are scheduled to attend the EEO courses in accordance with one of our AAP objectives. One Equal Opportunity Specialist progressed to the Management Analysis field.

Annual Report
Immunology, Allergic and Immunologic Diseases Program
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REPORT OF THE DIRECTOR

IMMUNOLOGY, ALLERGIC AND IMMUNOLGIC DISEASES PROGRAM

Fiscal Year 1979

A. Administrative Summary

1. Organization and Function

The Immunology, Allergic and Immunologic Diseases Program (IAIDP) was initiated on October 1, 1976, coincident with the reorganization of NIAID into major programs characterized by professional function. With this change the IAIDP assumed the responsibility for research, training, and conference activities that formerly were assigned to the Allergy and Immunology Branch of the Extramural Programs and contract activities in transplantation immunology previously administered within the Collaborative Research Program.

During FY 1979, the following organization served the IAID Program:

Office of the Director

Director-Sheldon G. Cohen, M.D.
Special Assistant to the Director-Dorothy D. Sogn, M.D.
Assistant for Program Management-George R. Jenkins, B.S.

Collaborative Projects Section
Head-Daniel I. Mullally, M.D., M.P.H.
Program Officer-John G. Ray, Jr., Ph.D.

Allergy and Clinical Immunology Branch

Chief-Robert A. Goldstein, M.D., Ph.D.

Immunology and Immunochemistry Branch

Chief-Bernard W. Janicki, Ph.D.

Genetics and Transplantation Biology Branch

Chief-Henry Krakauer, M.D., Ph.D.
Serum Bank Manager-Katherine A. Hopkins, R.N.

Each of the three IAIDP Branches assumes responsibility for administering investigator initiated research grants and collaborative research contract awards, developing programmatic initiatives and new projects, serving on trans-NIH coordinating committees and taking active roles in the development and conduct of workshops and conferences in areas of the biological disciplines and medical specialties with which they are concerned.

All three Branches are also involved with programs related to Research Career Development Awards and training, i.e., individual and institutional fellowships. In addition, the Allergy and Clinical Immunology Branch administers Allergic Disease Academic Awards which are designed to further the careers of mid-level investigators preparing for careers in Academic Allergy Research and the Young Investigator Research Grant Awards which are designed to assist and encourage investigators in early stages of their careers to develop research interests and capabilities in clinical aspects of immunology.

The IAID Program also assists investigators in their research by providing certain reagents that are not otherwise available from commer cial sources. Such reagents are intended either for reference standard materials such as human and mouse histocompatibility typing sera and various purified allergens. Program staff has the responsibility for maintaining adequate inventories of reagents as well as for developing new sources and identifying new materials for acquisition.

A new program activity, initiated in FY 1979 is designed to evaluate the efficacy of skin testing with major and minor determinants of penicillin prior to prophylactic administration of this drug or its analogues. Using multi-institution contracts for clinical testing data will be provided on the predictive value of newly developed diagnostic reagent material through the collaborative efforts of many physicians working in diverse clinical situations with patients manifesting a variety of infectious illnesses, some rare. It is anticipated that the use of common protocol with standardized material prepared and furnished to participating investigators will help to establish the criteria necessary for developing penicillin pre-tests as an important practical diagnostic tool and accelerate its clinical application. Of especial concern are the problems faced by patients with positive histories of penicillin reactions presenting indications for therapy with benzylpenicillin, ampicillin, carbenicillin, phenoxyethylpenicillin, phenoxymethylpenicillin and amoxicillin. Contracts for participation in this field trial were awarded to six geographically dispersed university sections having interdisciplinary programs in allergy and collaborative efforts with infectious diseases sections (Mayo Foundation; University of Rochester; Northwestern University; University of Colorado; University of Washington, and University of California at Los Angeles) and one interagency agreement was established with the Allergy Section of Walter Reed Army Medical Center. The Clinical Centers Program consisting of Centers for Interdisciplinary Research on Immunologic Diseases (CIRID) and Asthma and Allergic Disease Centers (AADC) continues to focus upon multidisciplinary collaboration, interactions between basic and clinical investigators and the application of research leads in the biomedical sciences to clinical problems in diagnosis, treatment and prevention. Project Directors have been exploring possibilities in their regional areas to increase the involvement of their staff in outreach areas of education, referral services and the socioeconomic aspects of disease and disability. The AADCs now total 16 in number with three new Centers added to this network, State University of New York at Stony Brook; for the study of urticaris, angioedema and vaculitis;

State University of New York at Buffalo; for the study of asthma, and University of Kansas; for the study of respiratory disease and systemic lupus erythematosus.

The rapid developments and progress in research on physiologic function, chemistry and genetics of immunocompetent cells have not only been important for the fundamental knowledge revealed but additionally have pointed to areas where clinical relevance may be. Accordingly, this year we have instituted efforts to facilitate interactions and exchanges between the Directors of NIAID supported clinical centers (CIRID and AADC) and Directors of Program Projects in Lymphocyte Biology. The effectiveness of this program has been sufficiently productive to move in the direction of expansion through the addition of one new "Lymphocyte Biology Center" at Jewish Hospital of St. Louis/Washington University, bringing the total of participating institutions to five.

Our mission as the designated lead Institute in the study of immune mechanisms has alerted us to the work of other NIH B/I/D concerned with organ and disease oriented interests involving dysfunctions of the immune system. Accordingly, we have developed joint program efforts especially related to conference activities and definition of research initiatives with NIAMDD (dermatology, diabetes), NEI (occular immunopathology), NCI (tumor immunology) and NHLBI (hypersensitivity lung diseases).

During FY 1979, the work of the Task Force on Asthma and Other Allergic Diseases was completed. One hundred and fifty contributors and consultants participated in the work of twelve panels. The final report of their Task Force dealt with the state-of-the-art and put forth specific recommendations for extended study in twelve specific areas concerned with social economic aspects, basic mechanisms, asthma, rhinitis, special problems of allergic children, farmer's lung and related disorders, the expanding problem of occupational/environmental asthma and related respiratory diseases; proposed development of integrated consultative and research facilities, dermatologic allergy, allergic emergencies, drug allergy, allergic and immunologic aspects of other diseases, and educational needs.

The Scientific Report has been published and distributed to the community of health professionals concerned withclinical and investigative endeavors in asthma and allergic diseases. An interpretative summary version is in the process of completion for distribution to the concerned lay community of health action groups.

Staff members of the IAIDP cooperated in the preparation of the updated Report of the Surgeon General on Smoking and Health. For this DHEW publication we assumed the responsibility of developing a state-of-the-art review concerned with the effects of tobacco and its products on the immune system in a chapter entitled "Tobacco - Allergy and Immunity".

The phase of data collection and correction of Kidney Transplant Histocompatibility Study (KTHS) has been completed and the first stage of evaluation and analysis is currently being carried out by the Naval Medical

Research Institute for the IAIDP. This work consists of characterization of donor and recipient populations, of selection, operative and post-operative procedures, of outcomes, and of certain elementary correlations among them.

The analysis of the data will be extended under contract with the Peter Bent Brigham Hospital, Doctor C.B. Carpenter, Principal Investigator, through a series of in-depth studies of specific issue in renal transplantation. These studies will be directed by groups of scientists representing the KTHS contractors and program staff.

2. Budget

The following shows the distribution of support by award mechanism for the activities of the Program during FY 1979.

Immunology, Allergic and Immunologic Diseases Program
FY 1979 Awards

Award Mechanism		Number	Amount
Research Grants		450	$ 46,195,862
Career and Career Development Awards		58	1,744,640
	Subtotal	508	$ 47,940,502
Fellowship Awards		42	539,221
Training Programs		34	2,311,604
	Subtotal	76	$ 2,850,825
Contracts		54	2,373,061
	Total	638	$ 55,118,775

[1]Total costs, estimated using a 40% overhead for indirect costs.

3. Conference Support

During FY 1979, the following meetings, conferences, and workshops relevant to the activities and functions of the Immunology, Allergic and Immunologic Diseases Programs were supported.

October 1979 Mediation of Effector Function by Antibodies.
Bethesda, Maryland

Immune Mechanisms in Renal Diseases,
Bethesda, Maryland

Conference on B Lymphocytes in Immune Responses,
Scottsdale, Arizona

November 1978 Asthma, Allergic and Immunologic Centers Workshop, Bethesda, Maryland

January 1979 The Role of Non-HLA Tissue Antigens in Human Transplantation, Bethesda, Maryland

February 1979 Review of Kidney Transplant Histocompatibility Study, Bethesda, Maryland

March 1979 Conference on Biological Recognition and Function, Keystone, Colorado

Conference on Cell Surface and Malignancy, Keystone, Colorado

The Regulatory Role of Macrophage Products in Immunity, Brook Lodge, Michigan

May 1979 Second International Lymphokine Workshop, Ermatingen, Switzerland

September 1979 Third International Workshop on Nude Mice, Bozeman, Montana

Workshop on Immune Mechanisms in Cutaneous Disorders, Brook Lodge, Michigan

B. Scientific Summary

Central to the activities and interests of the Immunology Allergic, and Immunologic Diseases Program (IAIDP) is the immune system, its functions in the maintenance of health, its involvement in immunologic and allergic diseases and its genetic relationships in transplantation phenomena and organ tranplantation surgery. Within the programmatic activities of allergy, diseases of the immune system and tissue transplantation is original work focusing on the structure and function and the basic biology of the immune system and its application to pathophysiologic changes. Responsibility for involvement in these biomedical areas is accomplished through investigator initiated research projects, collaborative research and development contracts, clinical centers concerned with asthma and allergic disease (AADC) and Interdisciplinary Research on Immunologic Diseases (CIRID), Lymphocytes Biology Program Projects ("Lymphocytes Biology Centers"), procurement and distribution of research resource and standard research reagent materials, and the maintenance of a serum bank for histocompatibility testing.

Designed to accomplish these objectives, the IAIDP is organized into three biomedical and scientific Branches: Immunobiology and Immunochemistry, Allergy and Clinical Immunology and Genetics and Transplantation

Biology. It is from the work of these three programmatic functional entities that the IAIDP has gained an insight into important developments and aspects of the state-of-the-art of both basic and clinical endeavors in immunology and allergy. A review of studies in immunobiology, immunochemistry and immunogenetics reveals not only progress in the generation of fundamental knowledge but also important potential applications of basic research data to problems concerned with health and disease through the function of the immune system. Investigations in asthma, allergic mechanisms, immunologic diseases, biology and inflammation, immunopathology and transplantation biology present promising leads for the prompt application of technologic advances to developing improved methods of diagnosis, treatment and prevention of relevant disorders.

An understanding of immunocompetency will depend upon the ultimate definition of the origin, differentiation pathways and functional roles of the various subsets of lymphocyte cell populations. It is therefore important to focus studies upon both the biology of progenitor cells and the identification of mechanisms of functional maturation. The most promising leads in this area are emerging from cell surface molecular markers on lymphocytes of both bone marrow and thymus derivation. Critical to the functional adequacy of differentiation and function of immunocompetent cells may be their specific enzyme contents. Keeping alert to expressions of defects in critical points of both T and B lymphocyte development may offer leads to deal with immunodeficiency diseases by appropriate reconstitution. The study and identification of cell surface antigenic markers, especially in embryos undergoing differentiation, can offer clues to immunologic association and involvement in the regression of cell maturation processes leading to neoplasia. Accordingly, it will be important to direct such studies to the investigation of both cell surface markers and cytochemical processes.

One of the most important benefits of the ascendency of research in cellular immunology is the increasing insight gained on cell to cell interactions. This is especially important in the potential application of incoming knowledge on both suppressor and helper influences of T lymphocyte cell subsets on antibody producing B lymphocytes in immune defense to microbial agents, hypersensitivity phenomena and transplantation reactions. Of especial pertinence is the regulatory role of products of the major histocompatibility complex on the functional role of other antigens expressed on the cell surfaces in the immune response. In searching for key factors for reconstitution in immune aberrations, a pertinent research approach which must be extended concerns new knowledge pointing to the necessity to match for functional interactions based on cells having same or self markers.

It becomes increasingly clear that immunocompetence depends upon a programed series of events among cells of different types to complete the process of recognition, uptake and processing of antigen and regulation of the critical signal to those cells involved in either humoral immunity through specific antibody production or cell mediated immune processes. However, it is not sufficient to recognize only physical contact between

macrophages and lymphocytes, and lymphocyte to lymphocyte of different subsets. It will be important to search out and identify cell-derived soluble factors that can be utilized in manipulation of the immune system. Central to this is the need to understand regulatory mechanisms whether through feedback or suppressor dysfunction in hopeful anticipation of the ability to design mechanisms and specific approaches to the control of both allergic and autoimmune diseases. Thus, such technologic developments that can lead to the separation and isolation of reactive cells with their subsequent cloning in culture are encouraging developments.

One of the early developments in the study of cell-mediated immunity was the identification of soluble products of lymphocytes termed lymphokines. Demonstrated only by their biologic activity in in vitro systems, this finding continues to give an implication of T cell reactivity apart from systems giving evidence of immunoregulatory functions. Though lymphokines have been purported to be responsible in a variety of immunologic reactions including inflammation, homograft rejection, delayed hypersensitivity, tumor and other cell killing, progress in this research area will require chemical identification. Another critical point is that lymphokine activity has thus far only been appreciated in in vitro systems. We will therefore be carefully looking at work that suggests the possibility of suppression of in vivo manifestations of cell-mediated immunity.

Though the direction of research endeavor in recent years has progressively moved to studies at the cellular level, important work continues on immunochemistry attempting to define the molecular components of the immune system utilizing chemical approaches. Supplementing earlier studies on the chemical composition and structure of humoral and secretory immunoglobulins, structure of antigens and the kinetics of immunocomplex formation are sophisticated investigations at the molecular level of cell surface components. Additionally, we see the emergence of a newly developing field, immunopharmacology, centering on the identification, characterization, isolation and attempted synthesis of chemical regulators of immune function. Elucidation of these factors can contribute to our understanding of immune dysfunction and measures to correct altered mechanisms in immunologic and allergic diseases. To understand the role of antibody synthesis in physiologic and pathologic situations, it will be important to have a clear definition of basis of antibody diversity. Accordingly, immunochemical studies offer the possibility for ascertaining whether antibody diversification depends upon a germ-line or a somatic process. An important indication is the likelihood that antibody diversity can arise by somatic cell mutation.

The past year has seen an escalation in technologic advances in an area bridging immunobiology and immunochemistry, especially concerned with the development of hybridomas. Cell fusion techniques and selection are being employed for the insertion of antibody producing characteristics into cultured malignant plasma cells so that by in vitro methods large quantities of pure immunoglobulins may be recovered. Another intriguing approach has been the development of methods for inserting functional messenger RNA into cultured cells. By these techniques, investigators now

may be able to obtain an unlimited source of homogenous antibodies and other immunologically-important molecules for study. The application of recombinant DNA technology to the problem of suppling large amounts of immunologically active reagents also is feasible and is being watched with considerable interest.

Another technologic advance has been the development of a fluorescence-activated cell sorter which can be utilized to prepare pure populations of human mononuclear cells from peripheral blood for functional studies. In addition to the contribution that this methodology has made to immunobiologic studies it may be utilized in the detection of neoplastic lymphocytes types and, in turn, be utilized to monitor therapy in patients with lymphomas.

The major objective of research in immunogenetics is the acquisition of sufficient understanding of the immune processes that lead to acceptance or rejection of a graft to render human organ transplantation a successful therapeutic modality. Indeed much of the knowledge of immunogenetics has resulted from attempts to define the genetic and antigenic relations that determine histocompatibility, i.e. from "tissue typing."

In all the mammalian species studied, histocompatibility is governed principally by an array of genes in a well-delimited region of a single chromosome, the so-called major histocompatibility complex (MHC). It is designated H-2 in mouse, the most exhaustively studied animal, and HLA in man. The genes of the MHC code for a series of cell surface antigens, most of which have been identified by serologic techniques as a result of their ability to stimulate antibody production and some by their ability to induce the proliferation of nonsyngeneic lymphocytes.

The genes of the MHC are very highly polymorphic. In man, the two most extensively studied loci are designated A. and B. An ever growing list of serological specificities, presumably reflecting antibodies against specific antigens, currently about 70, have been identified. Until recently, matching to attempt to obtain histocompatibility in organ transplantation was restricted to these loci. Such matching has very substantial predictive power value for renal graft acceptance when donor and recipient are related. Whether matching at the A and B loci improves graft survival when donor and recipient of the kidney are not related is not at all clear, especially in the highly heterogeneous U.S. population. In the case of transplantation between related persons, matching at the A and B loci implies very strongly matching over the entire MHC. In man, at least three other loci have been identified. The C locus codes for serologically determined antigens, but has relatively little effect on graft survival. The D locus codes for antigens determined by cellular methods: lymphocytes disparate at the D locus stimulate each other to proliferate. Matching at the D locus is particularly critical in bone marrow transplantation. Indeed, the mixed lymphocyte reaction involving cells of donor and recipient may be characterized as a transplant in the test tube. It is usable in renal allografting from a living donor, but, because of the time it currently requires, it can not yet be used when the donor is a cadaver.

Under intense investigation at the present time through an International Workshop sponsored by NIAID (IAIDP) is the role of the DR locus which is very closely linked to the D locus in renal transplantation. The initial reports from Europe are very encouraging, but the data beginning to become available on the experience in the U.S. are much more ambiguous.

Overall, it is clear from work in human organ transplantation as well as in animal models that matching at the MHC does not guarantee graft survival even when donor and recipient are related, unless, of course, they are identical twins. There are many other loci on various chromosomes which code for cell-surface antigens which, should they be polymorphic, could function as histocompatibility antigens. In addition, evidence for tissue-specific alloantigens is accumulating. Current histocompatibility testing focuses on lymphocytes as the antigen-bearing cells. The expression of antigens on various tissues is not uniform. The existence, therefore, of tissue-specific histocompatibility antigens is to be expected. It does, however, increase the difficulties of matching donors and recipient very substantially.

Alternative maneuvers are being attempted to improve graft survival. Among selection procedures there are tests of general reactivity of the recipient to antigenic stimulation. For example, the vigor of the response to challenge with dinitrochlorbenzene prior to transplantation correlates significantly with the vigor of graft rejection. Pretreatment of the cadaveric kidney to reduce its immunogenicity is under active investigation. Preculture of thymic cells and of pancreatic islet cells has been found to sufficiently enhance their survival after implatation. There are now numerous reports of the beneficial effects of blood transfusions on the survival of renal allografts, whether from cadaveric or living related donors. Our own Kidney Transplant Histocompatibility Study has shown that the much-feared consequence of blood transfusions, the sensitization of the recipient to a variety of donors, with the consequent increased difficulty of finding an appropriate donor, does not in fact occur with significant frequency.

Given the practical near impossibility of obtaining true histocompatibility, manipulation of the immune system of the recipient, and of the engrafted donor cells in bone marrow transplantation, is the sole remaining approach to maintenance of the graft survival. The mainstays of immunosuppressive therapy are adrenal cortical steroids and cytotoxic agents, principally azathioprine. Because of their toxicity, the transplanter treads a narrow path between graft rejection and patient death. A clear goal is the minimization of immunosuppression. There is thus a constant search for alternative strategies and more specific suppressants. One approach under investigation, with a clinical trial just now begun, involves massive irradiation of the recipient's lymphoid tissue with either sparing of the bone marrow or followed by transfusion with previously collected autologous marrow. The post-irradiated state in which the lymphoid organs are reseeded with lymphoid precursors is apparently one of great susceptibility to induction of tolerance, for example of a graft. A more selective immunosuppressant, under study for a number of years, is

anti-lymphocyte or anti-thymocyte globulin. The weight of the evidence is for its efficacy. Yet its effect is to improve graft survival only somewhat, and it is not certain that, like steroids and cytotoxic agents, it is not so generally immunosuppressive as to endanger the life of the transplant recipient without adequately enhancing the probability of graft survival. Thus, in one study, its abandonment led to improved patient survival without appreciable increases in graft losses.

A still more specific immunosuppressant, the cyclosporin family of drugs, whose target is a lymphocyte subpopulation, is now coming under careful study. There is very clearly great need for such highly specific agents, given the great complexity and delicate balance in the regulatory mechanisms that govern the immune response. It is of no use to so disturb these mechanisms with immunosuppressants as to produce an uncontrolled active response. Yet this does occur on occasion. It is, further, clearly necessary when intervening massively in the function of the immune system to carefully monitor the activity of its various regulatory components. The availability of reagents which identify various lymphocyte subpopulations that function as helpers, suppressors and effectors made this possible in the mouse recently. Similar reagents are now becoming available for man. Before long, the careful tailoring of immunosuppressive treatment of the transplant recipient should become possible. Together with careful selection to avoid, for example transmission of potentially lethal viral infections as in the case of cytomegalovirus with the transplanted organ and pretreatment of the recipient, solid organ transplantation should significantly improve its status as an effective form of therapy. Bone-marrow transplantation is considerably more difficult because, in addition to the possible rejection of the marrow by the recipient, there frequently occurs an immune reaction of the marrow against the recipient. Here also success is predicated on careful and selective immunosuppression. Significant advances are being made.

In recent years, numerous statistical associations between disease syndromes and HLA types have been established. These associations have practical diagnostic utility. However, much more interesting are the theoretical implications. In the mouse, genes that control response to various defined antigens have been mapped to the MHC. In addition, the serologically identified antigens have, in certain cases, been involved in the presentation of foreign antigen in the recognition phase of the immune response. Thus, there is promise that the association of the disease with a cell surface structure will lead to an understanding of factors that determine susceptibility to the disease.

While studies in basic immunology and immunogenetics have been responsible for rapidly expanding knowledge of fundamental biologic processes, at the same time they have been increasingly generating data to provide insights into mechanisms of allergic and immunologic diseases. During recent years the escalation of rewarding productive investigation into allergy, clinical immunology and immunopathology has been a direct product of a maintained awareness of the potential of basic experimental leads and our ability to seek their application to important clinical problems. An

understanding of etiologic and pathophysiologic factors of disorders of the immune system in turn has changed the direction of our approaches to work directed to the development of improved methods of diagnosis, prevention and treatment of allergic and immunologic diseases. Research focused on pharmacologic, biochemical, and molecular aspects of immunology by elucidating of functions and abberations of the immune system is providing the necessary base of information for promising approaches to deal with hypersensitivity, inflammatory and immune deficiency disorders by immune manipulation and immunopharmacologic techniques.

A number of the multifactorial influences contributing to the expression of asthma as a clinical disorder are being more clearly defined. Along with the established role of inhalant and food allergens there is increasing evidence of the sensitizing role being played by chemical and viral agents. The multiplicity of allergenic, inflammatory and infectious etiologic entities appear to have a common denominator of pathophysiologic action effected through the release of chemical mediators such as histamine, slow reactive substance of anaphylaxis, eosinophil chemotactic factor and platelet activating factor. It is expected that ongoing immunogenetic studies will help to explain the mechanism of genetic predisposition to allergic involvement of the respiratory tract and other target organs of allergic processes.

Because asthma continues to be an incompletely understood disease complex, it is necessary to direct attention to investigative approaches from several different standpoints. Among pathologic factors to be delineated are the physiochemical character and control of mucus secretion, the protective role and possible alterations in bronchial mucosal barriers and bronchial epithelial permeability, and structure and function studies involving mast cell distribution and smooth muscle of the bronchi. Since neurogenic influences play an important role in the control of bronchial smooth muscle tone, it will be important to more clearly establish contributions by improperly functioning autonomic nervous control and excessive cholinergic activity, nonadrenergic inhibitory nervous system influences in the tracheobronchial tree and whatever specific afferent fiber receptors may be involved in the physiology of reflex mechanisms. Though smooth muscle spasm has been indicated as an important cause of airway obstruction in asthma, the relative roles of pathophysiologic involvement within the larger central airways or in the smaller periphreal airways are still to be determined. Developing improved methods for the treatment of asthma will depend upon information that can be obtained from extended approaches on the study of the pathophysiology of airway obstruction, the neurogenic control of bronchial smooth muscle and the pharmacodynamics of asthma. In the area of diagnosis we are looking to the answers that will be provided by standardization of provocation testing based upon the challenges of exercise, tartrazine and aspirin inhalation, occupational exposures and pharmacologic agents. Studies under way which hopefully may contribute to preventive aspects include pathogenic factors of bronchial hyper-reactivity, food and environmental restrictions in infancy, the natural history of asthma, and the effect of immunotherapy on established asthmatic patterns.

Along with asthma there is evidence for immune mechanisms in a variety of other bronchopulmonary conditions associated with occupational and environmental factors, e.g., chronic bronchitis, fibrosis, granulomatous disease and hypersensitivity pneumonitis. Allergic reactions appear to be central to the pathogenesis of many types of occupational asthma and hypersensitivity pneumonitides. Among the isocyanates, (e.g., TDI), cotton linters and wood dust, experimental studies are focusing on a delineation of the respective irritant, allergic and pharmacologic pathogenic properties. In addition to occupational and environmental asthma, immune mechanisms are being shown to play important roles in the production of certain fibrotic and granulomatous lung processes associated with inorganic dusts, e.g., beryllium, silica and asbestos fibers. Important relevant investigations include those concerned with the study of basic immune mechanisms, clinical expressions, epidemiologic studies especially in the plastics, pharmaceutical and wood industries. It is anticipated that specially designed materials and methods for bronchial provocation, respiratory function tests, immunologic skin tests and serologic studies correlated with environmental monitoring will help us assess important causative factors and measures for their control. While the largest number of instances of hypersensitivity pneumonitis occur following occupational exposure, (e.g., moldy fodder in farmer's lung, moldy sugar in bagassosis, bird droppings in pigeon breeders disease, mold spores in maple bark stripper's disease) there is increasing evidence that sensitizing organisms contaminating forced air heating, humidification and air conditioning systems produce comparable pulmonary disease risks in homes and offices.

Important areas of ongoing research on hypersensitivity pneumonitis require the isolation of well defined antigens, controlled inhalation exposure, pathogenic mechanisms involving disposition of antigen, various parameters of the immune response, and immunopathologic and toxicity studies. Pertinent points for extended investigation will be focused on the immunochemical characteristics of suspected offending organic dusts and the definition of environmental factors involved in the generation and dispersion of such causative agents.

An understanding of immediate hypersensitivity will require a precise definition of structural molecular characteristics of basophil and mast cell receptors for IgE and the regulation of IgE synthesis and secretion. In turn, an important goal of research is the development of methodology to block IgE fixation and release mechanisms. In this connection studies of eosinophil biology and eosinophil-mast cell relationships can be expected to provide insights into the potential residing within such cellular mechanisms for modulation of hypersensitivity reactions and/or possible secondary cytotoxic tissue damage. Since mast cell hyperproliferation and eosinophil migration are prominent accompaniments of helminth tissue invasion, studies dealing with the immunologic and immunopathologic aspects of parasitic infection assume increasing importance.

Important areas of definition of basic immediate hypersensitivity mechanisms relate both to chemical mediators of inflammation and interactions between immunocompetent cells. Approaches to developing

pharmacologic agents capable of preventing or modulating allergic inflammation have been aided by emerging information on the two types of histamine cell receptors (H1 and H2) and their specific antagonists capable of blocking this amine's bronchial vascular effects. Additionally, information on the surface characteristics of subclasses of lymphocytes may make it possible to selectively impair the function of suppressor, helper or cytotoxic lymphocytes and possibly to approach the treatment of both allergic and immunodeficiency disorders by immune modulation or reconstitution.

In the area of drug reactions we are looking to the possibility of providing tests of predictive value in allergic reactivity. Additionally, the morbidity due to allergic drug reactions could be considerably reduced by attention to such factors as isolation, purification and possible elimination of the defined antigenic moiety. Identification of the actual chemical determinants for haptens responsible for drug allergy will assume increasing importance in our search for the development of specific diagnostic reagents in drug allergy situations. In addition to concentrating on immunological detection systems potentially capable of detecting cellular or humoral immunity applicable to drug sensitization an equally important approach is being directed to developing sensitive in vitro systems. Another approach which may eventually be helpful in reducing the incidence of serious drug reactions to specific drugs awaits ongoing studies on the identification of HLA or other genetic markers that may be associated with allergic reactivity based on altered drug metabolism, alterations in reactive metabolite information or innate immune responsiveness. Additionally important in the area of drug allergy are approaches to understanding the mechanisms of drug modulation of the immune response associated with exacerbation of autoimmune disease during the drug therapy. We also are looking to those studies on immunotherapy in allergic disease based upon the induction of tolerance as models for the design of tolerogenic analogues of therapeutic agents having allergic potential.

The importance of allergic mechanisms and immune system function has been demonstrated in several dermatologic disorders, especially atopic dermatitis, allergic contact dermatitis, urticaria and angioedema, the vesiculobullous diseases (pemphigus, dermatitis herpitiformis, bullous pemphagoid) necrotizing vasculitis and lupus erythematosus. Conversely, the study of these skin diseases has added considerably to our understanding of basic immune mechanisms. An appreciation of the ready access of skin for study of disease expressions in systemic immunopathologic processes merits our continued attention. We have been especially concerned with studies directed to identifying disorders and their mechanisms where skin may be primarily involved as target tissue by immunohumoral and/or cellular reactants, or where cells of the integument may serve as natural sources of sepcific antigens in immune processes. Important pathogenic mechanisms have been shown to include IgE involvement, activation of the complement cascade and formation and deposition of immune complexes. Atopic dermatitis once believed to be solely an immediate hypersensitivity disorder has now been shown to involve complex mechanisms including T cell abnormalities and possible immunodeficiency factors. As a

multifactorial disorder, work on this disorder is being concentrated on genetic predisposition and cyclic AMP cell biologic functions interacting with IgE mechanisms. In urticaria and angioedema where the role of chemical mediators of inflammation and vascular permeability can be demonstrated, the importance of complement activation and associated vasculitis are providing helpful approaches to the design of specific pharmacologic agents.

The importance of immune mechanisms has been demonstrated in a variety of connective tissue, renal, gastrointestinal, endocrine, hemotologic and neoplastic conditions. Among those of established definition meriting immunopathologic experimental approaches are rheumatoid arthritis, hepatitis, inflammatory bowel diseases (ulcerative colitis and Crohn's disease), intestinal malabsorption syndromes, neurologic disorders (Guillain-Barre syndrome, myasthenia gravis, multiple sclerosis), erythroblastosis fetalis, glomerulonephritis and interstitial nephritis, systemic lupus erythematosus, agranulocytosis, juvenile diabetes and T and B cell lymphomas. Over the past several years significant advances in basic immunology have exerted a major impact on our study and understanding of this broad range of diseases. Accordingly, many can now be classified into those related to specific immune mechanisms, e.g., immunodeficiency, immune complex deposition and autoimmunity. Accordingly, we are utilizing highly sensitive methodologies for the identification of etiologically important antigens, antibodies and immune complexes involved directly or indirectly in their suspected pathogenesis and techniques for identifying and fractionating various subpopulations of lymphocyte cells involved in immunologic reactivity and immunoregulation. Fruitful approaches include the identificaion of antigens and immune complexes in vasculitis related disorders and the identification of antibodies against cell surface receptors. The identification and functional delineation of subpopulations of lymphoid cells is expected to allow for a greater understanding of the primary and secondary immunodeficiency disorders and in turn an explanation of some immunoregulatory abnormalities in autoimmune diseases. We, therefore, continue to direct our programmatic concerns to technological advances emerging from basic research to provide useful tools for probing disease states and applying basic immunologic investigative data to clinically relevant disorders.

ALLERGY AND CLINICAL IMMUNOLOGY BRANCH

A. Scope

This Branch is concerned with the etiology, pathogenesis, diagnosis, prevention and treatment of both naturally occurring and acquired allergic and immunologic diseases.

Relevant studies of allergic diseases supported by the Branch include: (1) factors, both primary and predisposing, which contribute to the production of asthma, such as extrinsic allergy, infection, abnormalities of the sympathetic nervous system, cellular and chemical mediators of inflammation, and pharmacologic agents; (2) immediate type hypersensitivity and its disorders (allergic rhinitis, atopic dermatitis, urticaria and angioedema); (3) allergic phenomena affecting respiratory, gastrointestinal and cutaneous tissues; (4) allergic reactions and disorders caused by insect bites and stings, foods, airborne allergens, and infectious agents; (5) humoral antibody, particularly IgE, and the chemical mediators released by the interaction of antigen and antibody on target cells; (6) therapy and prevention of allergic disorders and hypersensitivity reactions by immunotherapy with specific antigens or drugs; (7) mechanisms of delayed hypersensitivity and contact dermatitis; (8) mechanisms of drug reactions and chemical sensitization; and (9) isolation and chemical characterization of the active fractions of known allergenic agents; and (10) epidemiologic and environmental studies designed to ascertain those agents or substances which may be of clinical relevance to allergic individuals--either as causal or contributory to their disease process.

In the area of immunologic diseases, the Branch activities are focused on studies of the underlying mechanisms of disease and the application of basic knowledge to the etiology, prevention and management of immunologic disorders. Either or both of two disciplinary approaches, Clinical Immunology and Immunopathology, are involved in this effort. Studies in Clinical Immunology are directed toward acquired and inherited diseases associated with dysfunctions of the immune system. Immunopathology studies include genetics, cytology, biochemistry, physiology and pharmacology of the immune system. Relevant areas supported by the Branch include studies of (1) immune deficiency diseases arising from primary defects in development or maturation of the immune response or secondarily resulting from disorders affecting immune responses, (2) clinical manifestations mediated by products of lymphocytes, (3) diseases associated with immune complexes and autoimmune phenomena, (4) immunodermatology, i.e., immune disorders involving the cutaneous system, and (5) immunotherapy of disease processes.

The ultimate goal of the Allergy and Clinical Immunology Program is to promote the acquisition, translation, and application of research findings to the diagnosis, prevention, and treatment of allergic and immunologic diseases. To achieve this goal, the Branch is responsible for and manages Programs of Institute Emphasis (PIE) in Asthma and Allergic Diseases, Immunologic Diseases, and Immunology Centers and Program Projects. Collaborative clinical as well as basic research studies are supported among Centers and Program Projects in an effort to take advantage of the

nationwide character of these programs and to facilitate achieving our goals. To support these efforts, the Branch funds targeted contract efforts and also obtains and distributes selected research reagents and materials to investigative allergists and clinical immunologists.

B. Awards and Support Levels

Allergy and Clinical Immunology

FY '79 Awards

Award Mechanisms	Number	Amount[1]
Research Grants	151	$ 15,930,164
Career Awards and Career Development Awards	21	783,711
Subtotal	172	$ 16,713,875
Fellowship Awards	16	216,015
Training Awards	24	1,611,585
Subtotal	40	$ 1,827,600
Contracts	10	452,156
Total	222	$ 18,993,631

[1]Total Costs, estimated using a 40% overhead for indirect costs.

The distribution of these awards by area of study is approximately 30% for asthma and allergic diseases and 70% for immunologic diseases. Approximately 34% of these awards were for competing new or renewal applications; the remainder represents commitments to support awards made in prior years.

Support for the PIE activities is included in the research grant category. During FY '79, the Branch supported these activities with awards for 15 Asthma and Allergic Disease Centers at a total cost of $1,787,467, 6 Clinical Immunology and Immunopathology Program Projects at a total cost of $3,093,499, and 4 Centers for Interdisciplinary Research in Immunologic Diseases at a total cost of $1,184,446.

C. Program Areas and Highlights

1. Asthma and Allergic Disease Centers Program (AADC)

A network of 15 Asthma and Allergic Disease Centers, located throughout the United States, is actively engaged in a collaborative fashion to gain new knowledge and insights into the field of allergic disorders including those manifestated in the upper and lower respiratory tract, skin, and other organs such as kidney, gastrointestinal tract, etc. Research, both fundamental and applied, is conducted; and through this multi-faceted approach, methods have already been developed to improve the diagnosis and treatment of asthma and other allergic diseases. Although

the emphasis of the Centers Program is directed toward a better understanding of the basic mechanisms involved in the various allergic disorders, they also have provided the field of allergy with unique academic resources in the discipline of allergy.

By serving as referral centers for patients in their areas, by engaging in collaborative clinical trials to assess diagnostic and therapeutic discoveries, and finally by acting as an important resource for training academic allergists for the future, they have had a profound impact on the delivery of health care to allergic individuals. Separately each of these Centers have also been successful in competing for additional support through either the regular grant program, training grants, or research career awards.

At the Eighth Annual AADC Workshop in Bethesda on November 30 to December 1, 1978, directors and professional staffs from the 15 Asthma and Allergic Disease Centers (over 125 participants) met at NIH to review progress by grantees in this Centers Program. While the presentation of scientific papers (more than 50) provided the focal point of interest, the ultimate success of this workshop can be measured better by the broad and open discussion and the significant opportunities for investigators to exchange ideas for future study. A measure of the importance of this meeting came from the overwhelming requests by people outside the Centers Program to participate.

Brief highlights of some of the activities conducted by our Asthma and Allergic Disease Centers follows.

John Salvaggio, AI 13401, Tulane University and his colleagues have focused on Environmental/Occupational Hypersensitivity lung diseases. Purification of coffee and castor bean allergens enabled them to perform studies showing that workers in the coffee industry were sensitized by exposure to sacks that had previously been used to transport castor beans. Persons with asbestosis and silicosis have been found to have a high frequency of antinuclear antibody, a finding which may lead to better understanding of the pathogenesis of fibrotic lung disease. Finally, studies of mold sensitive asthma have progressed with clarification of antigenic relationship among 3 species of aspergillus--an important effort in attempting to clarify and control environmental sources of these allergens.

Roy Patterson, AI 11403, Northwestern University has continued efforts to reduce the allergenicity of ragweed antigen E while retaining antigenicity by use of polymerized fractions; preliminary clinical trials with this material has demonstrated efficacy when compared with monomer preparations but with greater safety. Expanded trials in collaboration with other centers are underway.

Richard Hong, AI 10404, University of Wisconsin and colleagues have continued studies investigating the role of viral infections in predisposing or causing asthma.

Oscar L. Frick, AI 11010, University of California at San Francisco similarly has been in following children prospectively in order to clarify the meaning of a coincidental association between certain virusinduced colds and flu-like symptoms and the onset of allergy during the first year of life. They found in a small group that antibodies to influenza-like viruses and certain environmental or food materials (dog, cat, house dust, grass or cow and soy milk) increased at time of onset of allergic symptoms. Studies were conducted to learn whether certain viruses can act to alter the immune system so as to trigger the onset of allergy.

Kenneth P. Mathews, AI 10391, University of Michigan has continued studies of "vasomotor rhinitis" in an effort to define the varied mechanisms by which people may be affected. Clinical trials are also being conducted assessing efficacy of intranasal treatment with gluteraldehydeaggregated ragweed extract in treatment of ragweed hay fever--which if successful could greatly reduce morbidity and cost to patients.

Philip S. Norman, AI 10304, Johns Hopkins University and colleagues have continued studies of modified allergens or allergoid (formaldehyde modified whole extract and allergen). Also they provided fundamental data which permitted commercial release of venom extracts in prevention of anaphylaxis from bee stings. A double blind controlled trial is in progress to study the effects of Rinkel immunotherapy--no difference has been found to date between this and standard immunotherapy.

Gerald J. Gleich, AI 11483, Mayo Foundation and colleagues, taking advantage of ongoing studies to refine allergens and develop sensitize diagnostic tests, have shown that efficacy of ragweed pollen immunotherapy is associated with reduction of activity of IgE antibodies in the radioallergo-sorbent test, thus giving a means of precise monitoring of efficacy.

2. Centers for Interdisciplinary Research in Immunologic Diseases (CIRID)

In September 1978, 4 Centers for Interdisciplinary Research in Immunologic Diseases were funded by NIAID. This program is designed to foster integration and coordination of research projects in clinical immunology being pursued in other clinical specialties (e.g. dermatology, pulmonary medicine, hematology, nephrology, rheumatology, infectious diseases and otorhinolaryngology) with those in basic research (e.g., immunochemistry, microbiology, virology genetics, biochemistry, pharmacology, general physiology, and pathology). An important additional component of these Centers is the funding of specific projects' in demonstration/outreach activities, which are designed to impact on the health care of the community in a tangible and immediate way by bringing research advances directly to the patient. While it is obvious that during the start up year efforts are of necessity preliminary in nature, the following is intended to provide an idea of how progress is anticipated in this area.

At UCLA (John Fahey, AI 15332) an evaluation of a supplementary program of nonmedical intervention on the course of childhood asthma is being conducted by (E. Lewis, M.A. Lewis & G. Rachelfsky). A curriculum has been designed to teach children and their parents what asthma is, to recognize

initial symptoms, and then to utilize self-management techniques such as relaxation and breathing exercise to terminate or minimize each episode; to recognize indications for use of certain pharmacologic agents; and finally to conduct field trials. Early evidence from an initial group of 10 children and their families suggest that the desired level of information can be acquired and more importantly that families are enthusiastic in their participation.

John Leddy, AI 15312, University of Rochester has designed and implemented a 12session concentrated course in Clinical Immunology for physicians and other medical personnel (e.g., nurse practitioners) affiliated with outlying community hospitals in the large semi-rural region served by the University of Rochester. It is hoped that this will lead to better comprehension and therefore diagnosis and treatment of immunologic diseases in rural upstate New York. To prove this point a means to assess the benefit to the patient will be undertaken not only by sequentially testing participants, but also by tracking referrals and as well open lines of communication fully between the CIRID and the regional community.

Joseph Bellanti, AI 15321, Georgetown University and his collaborators have sponsored a symposium on public concerns of immunization (October 1978) as part of a broader effort to facilitate the transfer of basic science knowledge to the general public. They have also initiated an Asthma Education Intervention Project emphasizing self-management techniques for pediatric asthmatics and their parents. They have further initiated study of prevalence of asthma and other allergic/immunologic disorder and cost and utilization of health services in the metropolitan Washington, D. C. area.

Charles W. Parker, AI 15322, Washington University St. Louis has initiated a program whose intent is similar to that of the Rochester CIRID, in that it is designed to enhance knowledge of immunologic disorders in the urban community served by the CIRID.

It should be mentioned in closing that while this report has emphasized the outreach/demonstration activities of the 4 CIRIDs, the largest proportion--more than 90 percent--of their efforts continue to relate to basic and clinical research.

3. Program Projects in Mechanisms of Immunologic Diseases

Program Projects were established to encourage the development of collaborative basic science and clinical investigation in immunologic diseases. As such this program is designed to further investigate underlying mechanisms of disease and to enhance basic knowledge relevant to eiology, prevention and management of immunologic disorders. Studies are effected from either one of two disciplinary approaches: clinical immunology or immunopathology. Clinical immunology studies are directed toward acquired and inherited diseases associated with dysfunctions of the immune system. Immunopathology studies include specific areas of genetics, cytology, biochemistry, physiology, and pharmacology of the immune system and its disorders.

In FY 1979 there were 7 active grants in this program. The following will be an attempt to present sufficient detail from each to show the depth and breadth of this program.

The project headed by Frank J. Dixon (AI 07007 - Scripps Clinic and Research Foundation) is designed to gain a better understanding of the molecular basis of diseases caused by aberrant immune responses. Its focus is upon 1) normal controls of immune responses, 2) the means by which unusual presentation of an antigen, particularly associated with chronic viral infections, may initiate pathologic responses, 3) the chemistry and function of the several mediator systems which are activated by immunologic reactions and cause injury, and 4) the multiple complex immunopathologic events found in spontaneous or experimental immunologic disease. The diseases most immediately related to this research include glommerlar nephritis, systemic Lupus erythematosus, rheumatoid arthritis, other autoimmune diseases, acute and chronic viral infections, allergic and anaphylactic reactions, allograft rejection, and disorders of complement and Hagemann factor occurring in mediator systems.

Measurements of immune complexes and biologic fluids appear to be providing useful information helpful in monitoring the clinical courses of patients and guiding therapy. It is hoped that isolation of immune complexes may contribute to the identification of pathogens and diseases of unknown etiology, and to the development of practical diagnostic and therapeutic measures. An example of the value of such studies relates to the belief that the immune complexinduced degenerative vascular lesions seen in murine Lupus may have relevance to degenerative arterial disease in man; supported by the knowledge of increasing occurrence of coronary artery disease and myocardial infarction in humans with Lupus, confirmed by preliminary studies in autopsies, as well as examination of the deposition of immune complexes in arterial walls. It is thus thought that repeated vascular deposition of immune complexes associated with incidental immune reactions might provide a significant component in the genesis of atherosclerotic disease.

Studies by Joseph Feldman as part of this program project have determined that survival times of skin grafts, repeatedly applied to BN and Lewis rats, were unaffected by the levels of circulating immune complexes, and in rats grafted with allogeneic kidneys (vascular graft) and in dams repeatedly impregnated, no circulating immune complexes were detected. The signifance of these studies is related to the desire to examine the hypothesis of the aging process and neoplasic (which occur with increased frequency in aged hosts) may well be associated with some membrane changes (microviscosity, cholesterol, phospholipid) and with cytoskeletal membrane interactions that revealed deficiencies in immune surveillance and response.

In vivo studies by Charles G. Cochrane have examined the participation of the Hagemann factor system and complement component in the development of immunologic injury, which were made possible by biochemical analyses of the individual components and their mechanisms of action. With the availability of components of the Hagemann factor system in both rabbit and human, together with methods for their detection, definitive experiments

were made possible to detect the active participation of such components, thus indicating that any deficiency in this pathway will abrogate humoral mediated destruction of virus-infected cells.

Studies by Curtis B. Wilson have concentrated on examining the role of monocytes and macrophages in immune-complex induced glommerular nephritis in rabbits. An extension of previous studies regarding the contribution of monocytes and macrophages have shown that monocytes and macrophages can be recovered in large numbers by tissue culture of isolated glommeruli taken from rabbits with the most proliferative forms of experimental serum sickness glommerular injury. It is hoped that results of these studies will enable characterization of differing patterns of immune complex localization in kidneys and efforts have been made to relate these to host genetic factors which could influence the susceptibility of individuals to immune complexinduced glommerular injury.

William O. Weigle has continued studies examining the activation of T and B cells and the interaction of those cells with macrophages in the induction of immune tolerance and autoimmunity. These studies led to the description of a model of immunologic tolerance to human gammaglobulin which is maintained in the absence of demonstrable suppressor cells. Other studies showed that the tolerance state induced in B cells in adult mice to human gammaglobulin is the result of central unresponsiveness rather than of antigen blockade. A thymus replacing factor (TRF) has been produced by the activation of spleen cells with concanavalin A, the cell type in the spleen responsible for the generation of thymus replacing factor is a T cell, that is, LYT 1+ and LYT 2-. Preliminary studies indicate that TRF obtained from mice sensitized with sheep red blood cells support a primary response in nude mice. Further studies by Hans Muller-Eberhard into the mechanism and functional role of complement have shown that the isolated component mixture containing the proteins of the alternative and of the membrane attack pathway is capable of generating bactericidal activity, thus showing that the system is complete and antibody is not required for alternative pathway initiation. These observations relate the cytolytic alternative pathway to natural resistance to infection. Thus, in vivo, although bacteria are usually phagocytized and killed intercellularly, complement is required for opsonization (C3B) and to some extent for intracellular killing (C5 through C8). Individuals with homozygous deficiency of one of the precursor proteins of the membrane attack complex have, as expected, an increased susceptibility to infections, particularly of the Neisseria type. Thus, these reported structural and functional analyses of the membrane attack complex have provided a basic understanding of the manner in which complement lysis envelope the viruses and kills bacteria in animal cells.

Hugh O. McDevitt, AI 11313, Stanford University has been engaged in an overall effort to examine Humoral, Cellular and Genetic Mechanisms in Autoimmunity. Studies concerned with the relationship between the major histocompatibility complex (MHC) have demonstrated the presence of immune complexes in patients with two related forms of arthritis, ankylosing spondylitis and Reiter's syndrome. The suggestion is made that these patients may have an abnormal immune response to certain bacterias, which

leads to formation of immune complexes which may be the cause of the arthritis. Other diseases being studied for possible genetic susceptibility include rheumatoid arthritis, multiple sclerosis, diabetis mellitus, and systemic Lupus erythematosus. Further studies aimed at improving MHC typing are being conducted by F. Carl Grumet in this program.

A. Cacin has been following seroepidemiologically persons with enteric infection and Reiter's syndrome, finding that Shigella flexneri 2a and flexneri 1 cause Reiter's disease.

Samuel Strober has been conducting studies assessing the use of total lymphoid irradiation in treatment of auto-immune diseases and in the prevention of organ graft rejection. Four of 6 patients whose rheumatoid arthritis failed to respond to all conventional therapies had significant improvement in their disease activity which lasted from 6--18 months.

Roy Patterson, AI 11759, Northwestern University and his colleagues have concentrated their efforts on immune aspects of lung disease. Maintenance of a colony of rhesus monkeys giving consistent asthmatic responses to ascaris as well as control animals has permitted investigation of response to prostaglandins and histamines as well as other allergic mediator substances. Studies of airways physiology as well as peripheral immune responses have been made possible. Dog models of allergy have also been utilized. Studies of human asthma have investigated physiologic responses to inhaled antigens and methacholine. Hay fever patients were found to respond normally in contrast to asthmatic subjects when challenged with methacholine; similarly however, they responded to asthmatics when challenged with antigen-this response was not mediated by cholinergic reflexes (atropine pretreatment made no difference). This group also has shed further light on the role of trimellitic anhydride (TMA) as an occupational hazard which may produce not only asthma but pulmonary infiltrative disease. Their development of accurate diagnostic tests will permit extension of these findings to a large number of potentially exposed workers.

Other investigations of environmental hazards have included the observation that di-2-ethyl-hexyl-phthalate was found to migrate from polyvinyl chloride tubing into human blood in dialysis patients. Studies are underway to learn whether this finding can be related to the clinical observation that such foreign substances could produce the accelerated form of atherosclerosis in dialysis patients or be responsible for other vascular damage.

Fred S. Rosen, AI 05877, Children's Hospital Medical Center, Boston and his coworkers have been examining the reactions and metabolism of immune proteins and cells. Continuing studies of genetic deficiency of complement components led to the description of the first homozygous (C3 deficient individuals and that C3 nephritic factor was an IgG molecule (an autoantibody to C3bBb). Further they documented a polypeptide produced in plasma of patients with hereditary angiodema, affects the postcapillary venule of man and is generated from the second component of complement by action of plasmin. Patients with acquired or common varied agammaglobulinemia were found to have no B cells or to have B cells that were

unreactive in the presence of lymphocyte mitogenic factor. Restoration of function in some of these individuals has been accomplished with use of steroids. Bone marrow transplants were found to restore function in Wiskott-Aldrich Syndrome and adenosine deaminase deficiency. Finally, the dermatomyositis-like syndrome of agammaglobulinemia was found to result from infection of the CNS with ECHO virus. It should be mentioned that all of these studies are being accomplished in the setting of a large referral center for study of immunologic disorders, especially in children.

David G. Marsh, AI 13370, Johns Hopkins University has continued studies of the Genetics of Immune Response in Man. Studying a group of 400 allergic patients and their families since 1971 has resulted in the observation of significant associations between specific HLA types and specific IgE mediated allergic responses to 4 different highly purified pollen allergens. In addition an IgE regulator gene, not linked to HLA, has been found to exert an additional control over specific IgE responses. Continued studies are planned to test the hypothesis that under immunogenically limiting dosage exposure, response to a specific antigen is regulated by relatively few immune responses (IR) and immune suppressor (IS) genes and that all IgE antibody responses may be controlled by a gene which regulates the overall synthesis of IgE to any specificity.

4. Mechanisms of Allergic Diseases

Studies within this program have been concerned with investigations of the etiologic factors, pathogenetic mechanisms, diagnostic measures, and therapeutic approaches related to the effective management of allergic disorders.

The release of inflammatory molecules from tissue mast cells is a necessary step in the evolution of allergic reaction of the immediate type. It is interesting to note that such reactions may also be relevant in other immune responses, such as those implicated in graft rejection, poison ivy and immune kidney diseases.

Timothy J. Sullivan, AI 10090, Washington University, in studies investigating the mechanism by which mediators are released from mast cells, has detected reactions which appear to control the release of histamine. The formation of 1,2 diacylglycerol (DAG) appears to be a key step, and drugs of the methylxanthine class have been shown to inhibit the formation of DAG and mediators in parallel. From these studies it is hoped that drugs can be developed to control mast cell mediated inflammation.

Kimishige Ishizaka, AI 10060, Johns Hopkins University, also looking at the mechanism of mast cell secretion, found that bridging of receptor molecules rather than polymerization of cell-bound IgE molecules was responsible for mast cell triggering.

Lawrence Lichtenstein, AI 07290, Johns Hopkins University, has shown that human basophils have an adenosine receptor which is linked to adenylate cyclase and increases cyclic AMP. When this receptor is occupied by adenosine, histamine release is stopped. Interestingly, this receptor

is antagonized by theophyllin, a drug commonly used for the treatment of allergic disorders.

Marshall Plaut, AI 12810, Johns Hopkins University, has studied the role of histamine type 2 receptors in vivo in man by use of the drug, cimetidine (an H2 blocker), and found no significant changes in either immediate type or delayed type skin reactions. Continued studies such as these should help clarify the role of mediators in acute allergic responses and further permit development of pharmacologic manipulation of these immune responses.

Stephen Wasserman, AI 00254, Harvard University, has shown that heparin is present in human mast cells and that in a rat model of allergic disease, mast cell enzymes have been found and released after immunologic challenge. These enzymes, beta glucuronidase and hexosaminidase, could destroy connective tissue and explain prolongation of problems in human allergic diseases. Finally, he showed that disodium cromoglycate, which prevents mast cell mediator release, proved effective in the treatment of systemic mastocytosis. Andrew Grant, AI 12621, University of Texas Medical Branch, Galveston, examining histamine release from basophils, has continued studies assessing the role of C5A, a small fragment from the fifth component of complement, which has been found to bind directly to the blood basophil, inducing release of histamine.

The role of reaginic antibody, also known as IgE, which circulates in the blood, is well established in the allergic response. Many investigators have been examining ways to determine how this immune response is regulated, in order to learn whether or not this response could be abrogated in some general way in order to benefit allergic individuals. For example, Kimishige Ishizaka, AI 11202, Johns Hopkins, showed that the IgE antibody response in the mouse is dependent upon the dose and nature of antigen--that is, induction of antigen-specific suppressor T cells is involved in the transient IgE antibody response found in the genetically high IgE responder animal.

Andrew Saxon, AI 15251, UCLA, found that control of B lymphycyte production was dependent upon the presence of T lymphocytes; that radiated T lymphocytes provided helper function without suppression of IgE at high T-to-B cell ratios.

Albert Kalisker, AI 13592, has shown among mouse strains normally insensitive to ragweed antigen, who have been treated with moderate doses of cyclophosphamide, results in an enhancement of their ability to produce high anti-ragweed IgE titers. By way of explanation, it is thought that the drug eliminated from those mice cells which were involved in the actual suppression of IgE production. Other investigators such as Rebecca Buckley, AI 12026, Duke University, has also been examining the role of suppressor cells in the IgE response. Samuel B. Lehrer, AI 14891, additionally has been assessing and detecting IgE antibody producing cells in different lymphoid organs, and selectively suppressing IgE production in the mouse with anti-IgE. Alec Sehon, AI 14526, has carried the studies of IgE to penicillin allergy, showing that suppression of IgE antibodies to a

constituent of penicillin, that is, the benzyl penicilloyl (BPO) group was induced by the administration of non-immunogenic conjugates of BPO with normal gammaglobulins of the corresponding species. David Katz at Scripps Clinic has shown that IgE levels can be altered by a humoral factor which may either suppress or enhance the igE response in genetically different animals. It is hoped that this may lead to an ability to alter man's response to external allergens and enable investigators to modulate the immune response in such a way as to abrogate any high Ige responders. If this material can be deemed safe for clinical trials, it could be an alternate and universal "therapy" for allergy.

Other studies related to IgE antibody include those of Frederick J. Grundbacher, AI 12360, Peoria School of Medicine, who has shown that low levels of IgA may constitute a predisposing factor to the development of allergies in children. Floyd J. Malveaux, AI 05385, Johns Hopkins has shown that the total number of IgE receptors per basophil is closely correlated with the level of circulating IgE. This relationship implies a genetic association between serum IgE and the number of basophil IgE receptors with a modulation of the number of receptors by circulating IgE.

Charles W. Parker, AI 10405, Washington University, has been studying the IgE receptor on the rat basophilic leukemia cell line, which has maintained in tissue culture. This receptor has been solubilized and purified and may permit possible design of analogs to this receptor which would compete with the receptor for IgE, thus inhibiting its ability to respond when confronted with an external allergen.

The interaction of IgE with mast cells and basophils has been amply demonstrated. Hans L. Speigelberg, AI 10734, Scripps Clinic and Research Foundation, has shown that IgE binds to a subpopulation of bone marrow derived lymphocytes. Peripheral blood contains approximately 4 percent IgE binding cells in contrast to tonsils and spleens which contain four to five times as many. While it is unclear as to the biologic role of the lymphocytes binding IgE, studies are underway to determine the number of IgE binding sites binding B cells in patients with allergies and other disorders.

5. Asthma and the Other Allergic Diseases

Studies within this program have been concerned with investigations of the etiologic factors, pathogenetic mechanisms, diagnostic measures, and therapeutic approaches to the effective management of various allergic disorders. Among some of the diseases covered are asthma, allergic rhinitis, hay fever, hymenoptera sensitivity, drug allergy, as well as basic studies in allergic dermatitis.

Approximately 9 million Americans have asthma, and although they may present with a variety of clinical presentations, in essence, it constitutes a disorder of reversible airways obstruction. This obstruction has three major elements: 1) contraction of bronchial smooth muscles, 2) increased or retained secretions of mucus, and 3) inflammation of the respiratory tract mucosa. In individual asthmatics attacks can be provoked

by exposure to specific allergens, and some of these individuals have other allergies such as allergic rhinitis. On the other hand, many asthmatics do not have a demonstrable allergy, and in these individuals a variety of infectious, nervous (adrenergic and cholinergic), genetic or inflammatory factors may play a primary predisposing or contributory role. The program on asthma is structured to investigate all of the major parameters contributing to describing the mechanisms of causation in this disease. Areas under investigation include: the natural history, allergens, broncho-provocation challenge, animal models, pulmonary function testing, chemical meditors, cell mediators, agernergic agonists and antagonists, parasympathomemedic agents, drug allergy, diagnostic methodology, and therapeutic agents.

Efforts to elucidate what causes asthmatic reactions in individuals have included studies like those of Stanley P. Gallant, AI 00304, University of Utah, who has examined the neutrophils of asthmatic individuals, showing that the number and quality of beta agernergic receptors to a variety of pharmacologic agents are essentially normal. Further dissection of the pathways of how while blood cells from asthmatic individuals respond should be helpful in elucidating means of pharmacologic action. The role of respiratory viral infections which induce asthma has continued under the leadership of Richard Hong, AI 10404, University of Wisconsin, whose collaborators have shown that respiratory virus infection, particularly with rhino virus, may produce transient airway obstruction similar to asthma, and that the white blood cells from these individuals with rhino virus illness do have an impaired inhibition of lysosomal enzyme release from neutrophils. These observations have been extended to influenza virus, which has been shown to induce changes in the intracellular levels of cyclic AMP. Hopefully, these findings may provide leads for using either measures of peripheral blood leukocyte responses as indicators of the asthmatic condition, or to monitor therapeutic modalities. Other investigators are similarly engaged in these kinds of studies.

During the past several years, broncho-provocation and inhalation challenge techniques have been refined to study how to induce asthmatic attacks, as well as being employed to corroborate the diagnosis of asthma and to ascertain the constituents of the chemical, cellular, and physiologic events in the ontogeny of the asthmatic response, as well as to evaluate pharmacologic agents.

Againdra Bewtra, AI 14233, University of Iowa has established that the sensitivity of airways to methacholine and histamine are comparable in asthmatics. Further studies have demonstrated that the chemical compound SCH-1000 protects against methacholine challenge on both the large and small airways; however, it protects against histamine only on the larger airways. Studies of responses to allergens are underway. Similar broncho-provocation studies have been conducted in asthmatic patients by Roy Patterson, AI 11759, Northwestern University with inhaled antigen, such as ragweed antigen E, as well as with methacholine. He has found evidence that asthmatic subjects appear to be more sensitive to inhaled antigen when tested after a period of natural environmental exposure than before. It has been impossible to date to correlate pulmonary function findings with

clinical results of immunotherapy. Since the inhalation of allergen per se can be a highly sensitizing event, as well as a provoking event, studies of broncho-provocation challenge with use of allergens have been impeded somewhat because of these undesirable side effects.

As an alternative, Dr. Patterson has been following a colony of asthmatic monkeys which appear to be to date the best animal model of asthma which may simulate the human disease condition, especially insofar as airways are concerned. For example, this antigen-induced asthma occurs only in selected monkeys, which like human subjects, have hyper-reactive airways. Dr. Patterson has shown that living antigen reactive mast cells reside free in the bronchial lumens of these monkeys, as well as in dogs and man, and that these cells can transfer the asthmatic type airway responsiveness to non-reactive animals.

A variety of chemical mediators, such as prostaglandins, histamines, and other agents, which have been postulated as being important in describing human airways responses, have been tested in this animal model and shown to simulate the human asthmatic condition. Such a model will provide for evaluating therapeutic agents prior to tests in humans and offers the unique opportunity to perform repetitive studies under controlled conditions that otherwise could not be undertaken in humans.

Additional basic studies which may prove relevant to the asthmatic condition include those of H. Benfer Kaltreider, AI 12296, University of California, San Francisco, who has studied immune functions of the lung, showing that sensitized lymphocytes capable of killing foreign cells could be induced to appear in lung tissue by either local or intraperitoneal immunization in animals. These killer lymphocytes have been found to be critical for the control of virally-infected or neoplastically induced cells. Additionally, alveolar macrophages may exert a suppressive effect on the responses of lymphocytes to several immune stimuli, and this appears to occur through substances such as prostaglandins, which may be released by the macrophages themselves. His efforts are at understanding the mechanisms of non-cellular and cellular suppression of local lung lymphocyte function, and thereby develop strategies for combating hypersentitivity reactions in the lung. Direct work studying alveolar macrophage function in asthmatic individuals is inhibited by the difficulties of performing bronchial lavage in individuals with hyperactive airways. However, studies are underway in several laboratories at this time, which should shed further light on these areas.

Similar studies of basic immune functions of the lung have been performed by Barbara A. Nichols, AI 00294, who has used the parasite model of Toxoplasma gondii to study the interactions between this parasite and lung macrophages.

Ragweed allergy is a disorder affecting approximately 15 million Americans. For most individuals it is a mild seasonal disorder commonly called hay fever, which is characterized by eye irritation and nasal discharge; symptoms are usually relieved by antihistamines; however, in some individuals, although not life threatening, are a source of considerable

morbidity. In others ragweed has been indicated as being causal in producing an asthmatic-like syndrome.

Most of the advances in understanding allergic rhinitis have occurred in this model, particularly in the areas of etiology, diagnosis, pathogenesis, treatment and prevention. Further progress can be expected to lead to greater understanding of factors involved in all allergic diseases.

Pertinent in this regard is the difficulty in obtaining purified allergen preparations. Despite the fact that there are at least 100 different proteins in ragweed, only five have been demonstrated to produce allergy in man. J. Donald Capra, AI 12796, University of Texas at Dallas has determined the chemical structure of the smallest of these five proteins, termed RA 5, and is completing studies on defining the structure of further components. More recent studies are making an effort to determine the amino acid sequence of cedar and sage allergens. T. P. King, AI 14422, has prepared conjugates of purified Antigen E with non-immunogenetic polymers. These conjugates of Antigen E show reduced allergenic activity, yet retain the immunosuppressive property of the native antigen, and would not have been possible without the clarification of antigen structure. He is continuing further studies in the immunochemical characterization of vespid venoms with similar goals.

The clarification of the structure of individual allergens is important both chemically and biologically. Commercial suppliers of pollen for use in human desensitization work may vary in their source, so if an individual is receiving treatments in various parts of the country, there may be increasing or decreasing response to treatment. In addition and more importantly, if an allergist clinician should change the supplier of his pollen extracts or if the supplier should obtain pollen from a different region of the country, the potency of the extract might be markedly different. Efforts are underway in this program to provide standardized antigen materials.

Gerald Gleich, AI 07047, The Mayo Clinic, has successfully separated the two chains of Antigen E, the major allergen of ragweed pollen. Biologic activity of the individual chains was preserved, and studies are underway to determine the amino acid sequence of each chain, including especially those portions of the chains which are the determinants of the allergenicity of the molecule. Presumably, if the ability to confer allergy resides in only a small portion of the molecule, then these studies could lead to a new and improved type of immunotherapy for ragweed pollen sensitivity. On the other hand, David Marsh, AI 09565, Johns Hopkins University, has focused his research on the highly basic proteins which are rapidly released from ragweed pollen grains, as contrasted with the acidic proteins, such as Antigen E, which are slowly released. Since allergenic reagents currently employed in immunotherapy contain a large propotion of acidic proteins, such as Antigen E, the addition of these rapidly released substances may lead to an improvement in immunotherapy.

Several investigators, including Roy Patterson, AI 11403, Northwestern University, and Philip Norman, AI 04866, Johns Hopkins University, have

pursued the development of chemically modified ragweed allergens termed allergoids. A variety of chemical agents (formaldehyde, urea, gluteraldehyde, ethylene, glycol, etc.) have been employed, but the main allergen studied in immunotherapy is the more rapid induction of blocking IgG antibodies with greater safety than conventional materials now in use. Larger amounts of immunogenic material can be administered in a shorter period of time without the risk of generalized allergic reaction. Clinical trials are underway to assess the value of these materials and they should be licensed shortly for more widespread use.

Other investigators such as Rebecca Buckley, AI 12016, AI 14314, and AI 70830, Duke University, have utilized the purified ragweed antigens, such as Antigen E, which are supplied by our institute's allergenic reagents program. With these purified materials, both Drs. Buckley as well as Dr. Patterson have been able to demonstrate the synthesis of ragweed specific IgE from lymphocytes cultured in vitro, thus establishing a laboratory-based system for further in-depth studies of human allergic diseases. Because of the need for more widespread use of purified ragweed antigens, combined with the depletion of existing stock, it became necessary for the Allergy and Clinical Immunology Branch to acquire new materials. T. P. King, AI 82567 Rockefeller University, has produced RA 3, RA 5, Antigen K and Antigen E. These materials will be available to investigators and insure maintenance of a supply of highly purified and well-characterized allergens for further studies. Exciting recent advances have been further carried forward in the field of the sting of insects of the bee family called Hymenoptera, which induce an overwhelming and sometimes fatal reaction in sensitive individuals. While firm data is lacking, it is believed that about 2 million Americans may be sensitive to these stinging insects, and although the number of fatalities each year is not precisely known, it is believed to be less than 500. For individuals who develop an overwhelming reaction to a Hymenoptera sting, an injection of epinephrine may be lifesaving. Since exposure to these insects may occur in areas where prompt medical assistance is not available, it was important to develop an effective preventive measure. The Hymenoptera include honey bees, yellow jackets, hornets and wasps. Their venoms contain several vaso-active amines and other noxious simple chemicals which are responsible for the immediate irritation experienced by anyone stung by these insects. In addition, their venoms contain several proteins, and it is these proteins which are responsible for the induction of the hypersensitivity state and the subsequent development of anaphylaxis in very sensitive individuals.

Until recently the only materials available for testing and treating allergic patients to insect stings were so-called whole body extracts which were prepared by grinding up insects. Through the collaborative efforts of Lawrence M. Lichtenstein, AI 02870, Johns Hopkins University and Philip Norman, AI 10304, Johns Hopkins University, it was established that whole body extracts available to practicing allergists contain little if any active venom protein, and that their studies showed that injection desentization therapy with these extracts have virtually no utility in diagnosis or prevention of insect sting allergy. Subsequently, these investigators obtained venom itself, prepared solutions for clinical

evaluation. They found an excellent corrolation between results of venom skin testing with clinical history of Hymenoptera sensitivity. When these same venom materials were administered in a series of immunotherapy injections to sensitive individuals, protection against serious reactions ensued in well over 95 percent of individuals. Other investigators have confirmed the efficacy of these materials, and in the spring of 1979, owing to their work, venom for desensitization was in fact made commercially available. Since this material has only been in the hands of practitioners for a short period of time, more data will be required to determine which individuals will require this form of treatment, further clarification of therapeutic regimens will have to occur, etc. What is clear, however, is that through clarification of the immunochemical properties of the active venom protein, efficacious immune therapy with this material has been possible, and the development of specific diagnostic procedures combined with skin testing, followed by immunotherapy in appropriate individuals, has resulted in protection in the vast majority of persons.

6. Immunodeficiency

The program on immunodeficiency is concerned with the etiology, ontogony, prevention, and treatment of structural and functional deficiencies of the immune system. Both naturally occurring and acquired disease states are included. Ongoing studies have focused on the congenital absence, failure of development, or other disorders affecting thymus or bone marrow cellular elements; abnormalities in production, inhibition or catabolism of immunoglobulin; deficiencies of specific complement components; and defective host defenses due to abnormalities of leukocytes. These naturally occurring or acquired defects of immunity provide a unique opportunity to expand the understanding of normal immune function. Only by its absence do we see a role for its presence in the sequence of steps which make up the normal immune response. Although these diseases are rare, the information gained is relevant to the general field of immunology and to many health problems including infectious diseases, allergy and arthritis.

Certain immune deficient subjects have been shown to demonstrate high serum IgE levels. Rebecca Buckley, AI 12016, Duke University, has examined the function of accessory cells such as macrophages and polymorphonuclear white blood cells in these patients, and compared them with allergic individuals. Abnormalities have in fact been shown in both groups of persons. Richard Hong, AI 14354, University of Wisconsin at Madison, has suggested that the exaggerated response in these allergic individuals may be related to a defect in T lymphocyte regulation. Dr. Hong has also studied the role of thymus epithelial cells in reconstituting certain immuno-deficient patients. He is examining means to improve methods of culturing thymus tissue prior to transplantation, and learn by in vitro methods whether or not this is of benefit to such individuals. He has found that thymus glands may be preserved if kept properly in an incubator, and shown that thymic transplantation can provide restoration not only for immune function in classically immune deficient individuals, but has extended studies into work in cancer patients who have secondary immune deficiencies. Bone marrow transplantation has accomplished cures in

individuals with aplastic anemia, as demonstrated by Robert A. Good, AI 11843, Sloan Kettering Institute for Cancer Research. In addition to generalized immune deficiencies, other important observations have been made which have permitted further extension in this exciting area of therapeutics. For example, Ralph Wedgewood, AI 07073, has shown that in the Wiscott-Aldrich Syndrome a combination of immune deficiencies may be observed, and further that a hereditary enzyme deficiency of adenosine deaminase could be reconstituted with use of bone marrow transplantation. Ronald C. Scott, AI 12617, University of Washington, by screening individuals who are immunodeficient for adenosine deaminase and nucleoside phosphorylase, has provided further insight into biochemical causes of childhood immune deficiency states.

Edward J. Moticka, AI 12191, University of Texas Health Science Center, Dallas, has studied the acquired immunologic incompetence which accompanies antigen-induced hypergammaglobulinemia. This interference with reactivity, an important regulatory mechanism for the immune system, may be responsible for the diminished immune capacity observed in patients with multiple myeloma.

Sandra S. Kaplan, AI 14218, University of Pittsburgh, studying the defect in polymorphonuclear leukocytes which occurs in the Chediak-Higashi Syndrome, which is almost uniformly fatal, has demonstrated that a defective blood platelet may be responsible for this susceptibility to infection because it fails to activate the white blood cell. Studies of drugs are underway to overcome this deficiency by activating leukocytes through biochemical changes similar to those mediated by normal platelets.

Harvey J. Cohen, AI 00311, Childrens Hospital Medical Center, Boston, studying children with chronic grammulomatous disease, who are known to be missing an enzyme in their white cells, has preliminary evidence permitting the prenatal diagnosis of this disease by study of fetal granular sites obtained by amniocentesis. This work emphasizes how the study of basic mechanisms may have a broad range of potential diagnostic and therapeutic applications.

Hugh Fudenberg, AI 13484, Medical University of South Carolina, has also been studying immune deficiency diseases and has described genetic markers on certain lymphocytes of patients with common variable agammaglobulinemia who develop a fatal kidney disease; and that IgA deficiency and X-linked hyper-IgM syndrome are heterogeneous diseases. Work on cystic fibrosis has shown a basic defect which may be related to a defect in alpha-2 macroglobulin in the serum.

7. Immune Complex Disease

Immune complexes result from the binding of antigens by antibodies. Subsequently, components of the complement system also become involved. Such complexes, therefore, arise during the course of the development of the normal immune response. Under some circumstances, however, these entities appear within lesions and indeed are necessary for the development. The pathogenetic mechanisms by which immune complexes cause tissue damage,

particularly in the auto-mmune diseases with which they are increasingly associated are still poorly understood. The lack of sensitive, quantitative assays for all types of immune complexes has impeded work in this area to some extent. Recent studies by Vincent Agnelo, AI 70968, New England Medical Center Hospital, who has refined two such assays, one for rheumatoid factor and anti-IgG antibody, and one for the C1Q component of complement, are helping to clarify these measures. An unexpected finding is that some rheumatoid factors crossreact with nuclear protein. A further advance by Douglas B. Cines, AI 05477, University of Pennsylvania was made by his utilization of a radio-immunoassay to quantitate IgG and the C3 component of complement. This technique can be used also to estimate the affinity of an antibody for an antigen (R. M. Rothberg, AI 07854, University of Chicago).

William P. Arend, AI 10975, University of Washington, has made an effort to determine what it is that renders an immune complex damaging. He reports that in rheumatoid arthritis, anti-IgG antibodies (rheumatoid factor) specific for the FC portion reacted with IgG to produce innocuous complexes, whereas anti-IgG antibodies specific for the FAB portion produced injurious complexes.

Systemic Lupus Erythematosus is an auto-immune disease in which anti-DNA antibodies play a permanent pathogenetic role. The cause for the appearance of the auto-antibodies is being studied in a genetically defined mouse strain which develops an SLE-like syndrome spontaneously. Defective suppression of the immune response in these animals can break the self-tolerance usually seen (M. Eric Gershwin, AI 00193, University of California, Davis). Many people are studying the strain and have shown that the induction of tolerance to DNA can be accomplished by treatment with appropriately constructed antigen. Bernard Stollar, AI 14534, Tufts University has undertaken studies to define the chemical requirements for developing reagents that can specifically prevent the formation of antibodies to nucleic acid. In detailed studies of the specificity of tolerance, in studies done in collaboration with Yves Borel, AI 13867, Boston Children's Hospital Medical Center, he has shown that tolerance to one nucleoside does not prevent the formation of antibodies to other nucleosides of DNA. This finding provides an approach to the study of the regulation of responsiveness and specific nonresponsiveness at the cellular level. The obvious hope in this regard is to develop information sufficient to permit appropriate manipulation of the immune response in order to effect therapeutic benefits. Thus, the development of specific immune tolerance would be a significant improvement over the use of non-specific immunosuppressive or anti-inflammatory therapy in the treatment of auto-immune disorders.

Immune complexes which may be also associated with chronic infectious diseases and cause inflammation and tissue damage are also found to be involved in graft rejection. That they may have a beneficial effect is suggested by the evidence that such immune complexes can modulate the response to the antigen contained within them, and in fact prolong graft survival. Thus, Joseph D. Feldman, AI 07104, Scripps Clinic and Research Foundation, and associates have been studying circulating immune complexes

which appear after graft implantation and their effect on the host immune response.

Many studies are now nationwide measuring serum immune complexes and trying to define their role in clinical disorders, whether they may be valuable in assessing diagnostic or other modalities. Continued emphasis by this program in developing expeditious ways whereby these measurements can be made standard and by which other investigators can compare data among several groups of individuals is of particular importance.

8. Inflammation

The process of inflammation is mediated by cells and by humoral factors. This process is essential for host defense against foreign substances. However, in certain disease states, inflammation plays a major role in tissue damage. An understanding of what initiates and what modulates inflammation is, therefore, crucial to explaining and controlling these disease states. The noncellular mediators of inflammation can be divided into two general categories, those of small molecular weight and those of high molecular weight. The group of serum proteins derived from the complement system and the associated enzyme systems of coagulation, fibrinolysis, and kinin fall into the latter category.

The initiation of the complement casade by either the classic or alternate pathway makes a major contribution to the development of the inflammatory response. Once activated, complement components have functions including opsonization, immune adherence, chemotaxis, cell lysis, and anaphylatoxin generation. Derangements of complement have been implicated in causing or contributing to a wide range of disorders including cancer, kidney diseases, collagen vasular diseases, infectious diseases, and inherited diseases.

Understanding of the complement system depends on isolation and identification of its components. Through work done by our grantees, it is known that there are approximately 20 complement components. Paul A. Liberti (AI 12365, Thomas Jefferson University) has devised a new isolation procedure for C12 which results in stable preparations retaining all native biological activities. He has used this procedure to measure the physical properties of C1q in order to determine its most likely molecular structure, thereby enabling them to relate its structure to function. Irma Gigli (AI 13809, New York University) has continued studies examining the structural and functional proteins of the complement system, and has shown that Ch serves an important function in the initial steps of complement activation and that C4bp (previously reported) functions in the control of the formation of C42 convertase, confirming the presence of a control mechanisms in serum which regulates the classical pathway of convertase. Shaun Ruddy, AI 13049, Virginia Commonwealth University, has shown that a newly discovered control protein, beta-1 H globulin, actually combine with one of the active complement proteins and blocks its function in producing inflammation. Understanding the mechanism of action should allow development of new methods to control inflammation induced by complement.

Particular interest has been placed on studies of the properdin or alternate complement pathway. The alternate pathway was, until recently, thought to have specific recognition proteins which bound to and thereby identified foreign particles for destruction. Hans Muller-Eberhard (AI 07007, Scripps Clinic and Research Foundation) reports that the complement protein, C3, has recognition function in the so-called alternate pathway. Antibody is not required for this reaction. A cell (fungal, bacterial, etc.) that is an activator of this pathway allows C3, from the plasma, to be deposited on its surface. The deposited C3 interacts with surface structures of activators such that a regulatory protein which ordinarily initiates inactivation of deposited C3, cannot reach it. As a consequence, the orderly assembly of complement enzymes procedes without interference by regulators and the cell is killed by the membrane attack complex.

Other studies of basic mechanisms have included those of Celso Bianco, AI 15221, S.U.N.Y. Downstate Medical College, who has examined the role of certain complement components in studies of macrophage motility. Preliminary experiments have shown that peritoneal macrophages respond to Bb, a cleavage product of factor B of the properdin pathway by spreading an inhibition of migration. Hans Muller-Eberhard, AI 13010, Scripps Clinic and Research Foundation, has also studied the responsiveness of human mononuclear phagocytes to activated factor B (Bb of the properdin system) showing that mononuclear phagocytes synthesize and secrete complement proteins, and that monocytes synthesize C5 which is intimately involved in processes which lead to activation of monocytes to spread in response to both Bb and anti-C5F (AB prime)2. Thus the Bb complement activation fragment influences monocytes, therefore creating a possible role for complement protein as mediators of cellular immunity. To whatever extent that it correct, it is of interest that this could have relevance in cancer study, and in fact, Alfred Esser, AI 14099, Scripps Clinic and Research Foundation, has shown that certain RNA tumor viruses can be lysed by human complement, supporting the proposition that complement can provide a major defense against infection with reto viruses (oncornaviruses, C-type viruses, mouse leukemia viruses which may be associated with the production of tumors in animals).

These basic studies have been very rewarding because they are useful in explaining mechanisms for several clinical problems. Understanding of these mechanisms is important for possible prevention and treatment of these diseases. For example, K. Frank Austen (AI 07722, Robert B. Brigham Hospital) reports that he has been able to work out the molecular structure and mechanisms of action of C3 nephritic factor, the abnormal protein present in patients with membranoproliferative glomerulonephritis. This protein causes the complement system to be fully activated in the serum of patients with various types of nephritis. This protein is a host antibody directed against protein of the alternate complement pathway and serves to stablize the interaction of the complement protein thereby circumventing all host regulatory mechanisms. This leads to virtually complete depletion of the complement system with resultant inflammatory reaction. He is currently working out a role of the alternate complement pathway in bullous pemphigoid, a disease in which the mast cell effector system also appears to be abnormal.

Other tantalizing clinical leads have come from basic research in complement. For example, Dr. Gigli states that recent studies have shown that the complement system may play an important role in the localization of myocardial infarction. Depletion of one of the components, C3, is accompanied by better outcome of animals in which experimental infarcts have been induced. Kenneth Mathews, AI 14950, University of Michigan, Ann Arbor, has described a patient with a deficiency of serum carboxy-peptidase B, which might predispose to radiographic contrast media reactions or chronic idiopathic urticaria or angiodema (worked on in collaboration with Tony Hugli, AI 15782, Scripps Clinic). The potential significance of this observation is that there is an implication that there is a subpopulation of patients who not only react adversely to radio contrast dyes, but who also may have the syndrome of chronic urticaria or angiodema, where serum carboxy-peptidase B deficiency may contribute to their clinical difficulties.

John P. Leddy (AI 12568, University of Rochester) has been able to correlate the inordinate white cell depression described after donation of white cells to the activation and consumption of the complement system. This white cell activation is coincident with the formation of some white cell depressing factor in blood passing through the nylon filter used during this donation procedure (leukapheresis). By determining the precise mechanisms for this problem it may in the future be eliminated.

The genetics of the complement system have been studied by various investigators. Harvey R. Colten (AI 12791, Boston Children's Hospital Medical Center) has been looking at congenital absences of complement protein in regard to increased susceptibility to infection, hereditary attacks of edema, and increased susceptibility to rheumatoid-like diseases. Chester A. Alper (AI 14157, Center for Blood Research) has found that the second complement component and factor B but not the fourth component are controlled by genes in the region near the HLA genes. In a more basic way, John Goldman, AI 12792, Michael Reese, has continued studies investigating the relationship of early components of complement to the H2 complex in mice, finding that functional levels of C1, C4, and C2 are under the control of genes within, or closely linked to the S region of the H2 complex.

A second category, that of low molecular weight mediators of inflammation, was previously mentioned. A discussion of basophils (or mast cells) must accompany any discussion of these chemicals, because these are the cells which release them. The sequence of events leading to mediator secretion is as follows: sensitization takes place; IgE attaches to the surface of mast cells and basophils; on re-exposure, the cell-bound antibodies are bridged by the allergen signaling release of the mediators from the cells.

Research in this area has been facilitated by several new advances: Herve Bazin (AI 12830, Catholic University of Louvain) through the development of a colony of rats with IgE secreting plasmacytomas, has made this immunoglobulin readily available; and both Dr. Parker and Dr. Ishizaka have established rat basophilic leukemia cells in culture.

Both of the latter investigators are also studying basophil receptors. Dr. Ishizaka has been able to demonstrate histamine release from these cells via divalent anti-receptor antibody without participation of IgE. Also studying these receptors in Floyd J. Malveaux (AI 05383, Johns Hopkins University) who has noted the relationship between serum IgE and the number of basophil IgE receptors.

While Dr. Ishizaka has shown that mast cells can discharge allergic mediators without involvement of IgE, Lawrence M. Lichtenstein (AI 11334, Johns Hopkins University) has demonstrated release of mediators from other than mast cells. Namely polymorphonuclear (PMN) leukocytes contain and release slow reacting substance of anaphylaxis (SRS-A) and eosinophilic chemotactic factor (ECF).

Philip S. Askenase (AI 12211, Yale University) is studying so-called cutaneous basophil hypersensitivity (CBH). Traditional views of delayed hypersensitivity and immediate hypersensitivity as being separate patterns of immune reactivity may no longer hold. For example, otherwise typical delayed reactions contain significant numbers of basophils. CBH can be transferred in animal models by either immune cells or serum. Similarly, the late IgE mediated reactions in man may be analogous to serum transfer of CBH.

Despite this clouding of hypersensitivity reactions, some discussion of what is known classically as the type IV hypersensitivity reaction--cellular hypersensitivity, mediated by T cells is needed. These T cells also function as cytotoxic effector cells, have a role in secreting mediators of delayed hypersensitivity, regulate immune responses as helper or suppressor cells and act as memory cells.

The first part of this discussion will be confined to studies of hypersensitivity relevant to clinical diseases. Contact dermatitis is a prototype for cell mediated immunity. Henry Claman, AI 12685, University of Colorado Medical Center) has used a mouse model to study the development of delayed hypersensitivity to dinitrofluorobenzene. This experimental model is important because the complete antigen made after contact sensitization is DNP-coupled-to-self, and thought of in this way, contact allergy can be considered a form of autoimmunity where at least part of the antigen is self. Vera Byers (AI 12947, University of California, San Francisco, is studying poison oak and poison ivy, two of the main causes of workman's compensation in California. She is developing systems to study the effects of these chemicals on human lymphocytes in vitro, in an effort to produce a state of unresponsiveness. Henry C. Maguire, AI 13337, Hahnemann Medical College and Hospital of Philadelphia, is also studying contact dermatitis. He is trying to determine the mechanisms of an interesting clinical observation the use of cyclophosphamide under particular conditions will increase the intensity of allergic contact dermatitis reactions. Fred S. Kantor (AI 060706, Yale University) is studying the opposite phenomenon--anergy. He has induced anergy in an animal model and found the effect to be mediated by a subset of T lymphocytes which adhere to nylon-wool columns.

When sensitized T lymphocytes are cultured in the presence of specific antigen, they release a variety of nonspecific mediators, or lymphokines, that act on other lymphocytes and macrophages among other cells. Manfred Mayer (AI 12371, Johns Hopkins University, has been doing basic research to determine what lymphokines are. In particular he has looked at lymphotoxin which plays a role in certain cytotoxic reactions mediated by lymphocytes, mitogenic factor, lymphocyte activating factor which is an amplifier of immune and allergic processes, and hydrophobic peptide. Louis W. Heck (AI 05207, Robert Beck Brigham Hospital, has been studying migration inhibitor factor. He has shown that macrophages, pretreated with proteinase inhibitors demonstrated an augmented response to subthreshold doses of MIF.

Eosinophils are known to be involved in inflammatory processes, particularly those allergic in nature. For this reason, some mention should be made of research in this area. Gerald Gleich (AI 09728, Mayo Clinic, has localized the basic protein of the eosinophil to the core of the granule. The clinical relevance of this finding is, as yet, unclear. Thomas T. Hubscher (AI 15001, Georgetown University) has discovered that eosinophils may serve to dampen the allergic response.

Macrophages which are differentiated blood monocytes differ from the other phagocytes in that they are long lived, are capable of continuing lysosomal enzyme synthesis, and may further differentiate into epithelioid cells which, in turn, may fuse to form multinucleated giant cells. The technology for these studies has been advanced by the work of Steven Douglas (AI 12478, University of Minnesota, who has developed a technique of freeze-fracture in conjunction with electron microscopy making it possible to study macrophage membranes at high resolution. In particular, he is interested in developing techniques for the study of the genetic regulation of their plasma membrane receptors for immunoglobulin. Victor Najjar, AI 09116, Tufts University, is studying a peptide called tuftsin which stimulates phagocytosis. He is looking at the possible curative ability of this compound in animals, especially those that have had their spleens removed.

GENETICS AND TRANSPLANTATION BIOLOGY BRANCH

A. Scope

This Branch supports and manages research on the immunologic factors that determine the acceptance or rejection of grafts and on the genetic relationships that underlie them.

The increasing prevalence of degenerative disease and of trauma that may impair the function of particular organs sufficiently to be incapacitating or life-threatening identifies the perfection of a successful transplantation methodology as one of the objectives of highest priority in current medical science. The technical surgical aspects have proved quite manageable but immunological rejection of the graft is the obstacle that has yet to be surmounted.

Research efforts supported by this Branch include:

1. Studies of strategies designed to minimize the likelihood of rejection by assuring the best possible antigenic match between donor and recipient.

2. Explorations of immunosuppressive regimens whose objective is to reduce the capacity of the host to reject the graft.

3. Studies of treatments of the graft that will reduce its ability to provoke a response.

Studies in animal models are supported that explore the genetic controls of the immune response, and, on a more practical level, test methodologies whose ultimate application is to man.

Genetically conditioned associations between antigenic characteristics and susceptibility to various diseases have recently been brought to light and also fall within the purview of this Branch.

Two important activities of the Branch are the management of the Kidney Transplant Histocompatibility Study which is now entering the analysis stage and the management of the HLA Serum Bank, a national cell typing resource for transplantation as well as basic research studies. Stocks of human B lymphocytes are being accumulated and eventually will be made available to the scientific community for use in immunological studies and histocompatibility typing. A comparable bank of mouse typing alloantisera is also maintained by the Branch.

B. Awards and Support Levels

The Genetics and Transplantation Biology Branch supports research activities through individual grants, research career development awards, fellowships, training grants, and very importantly, in conncection with its kidney transplant program and serum banks, through contracts.

The following shows the distribution of support for the activities of the Branch during FY 1979.

Genetics and Transplantation Biology Branch

FY'79 Awards

Award Mechanism		Number	Amount[1]
Research Grants		64	$ 6,531,867
Career and Career Development Awards		9	300,783
	Subtotal	73	$ 6,832,650
Fellowship Awards		7	78,760
Training Awards		1	12,298
	Subtotal	8	$ 91,058
Contracts		39	1,895,292
	Total	120	$ 8,819,004

[1]Total costs, estimated using a 40% overhead for indirect costs.

The majority of these awards, approximately 80%, are concerned directly with transplantation biology and the remainder support research on the genetics of the immune system and of disease associations. Approximately 30% of these awards were for competing new or renewal applications; the remainder represents commitments to support awards made in prior years.

C. Program Areas and Highlights

1. Immunogenetics

An immune response is the result of a complex set of events involving recognition of the antigen and of interacting cells of the lymphoid system and of macrophages. Such recognition is in all instances determined by the genetic constitution of the responder. On it depend the ability to resist infection, the ability to accept or reject grafted tissue, and susceptibility to diseases of currently uncertain etiology, many of which appear to have an immune component.

The pattern of recognition is predominantly, but not exclusively, determined by genes organized into a so-called Major Histocompatibility Complex (MHC) located, in all mammals studied to date, on a specific segment of a specific chromosome. The organization of the MHC is best understood in the mouse and in man. It contains genes that code for cell surface proteins that act as strong transplantation antigens because, if the compositions of these proteins are disparate in the donor and recipient of a transplanted organ or tissue, the graft is usually rapidly rejected. On the other hand, certain foreign antigens such as viruses must become associated with these structures to elicit an immune response in the host.

These major transplantation antigens have been recognized to be highly polymorphic both in studies on rejection of grafts and by their ability to stimulate the production of specific antibodies. Approximately 70 specificities have been identified at 3 loci in man on the basis of serologic reactivities, and the list continues to grow.

There are other polymorphic loci within the MHC, also definable, by the ability of their gene products to stimulate antibody production on alloimmunization. These antigens have been designated Ia antigens in the mouse and DR antigens in man. The loci coding for them are clearly identical with or very closely linked to genes which control several of the components of the immune response in the mouse and probably also in man. Thus, the I-A, I-B and I-C control helper functions. The I-C subregion also controls suppressor functions related to the mixed lymphocyte reaction (see below), while the IE subregion codes for antigens on T cells capable of generating soluble suppressor factors. Helper and suppressor functions of T cells are also identifiable by means of the Ly cell surface proteins, which, however, appear to represent a separate system of differentiation antigens.

Other regions within the MHC control the ability of lymphocytes to proliferate in response to exposure to other lymphocytes from a non-syngeneic donor, ultimately producing progeny capable of lysing the stimulator cell. The proliferative response is called the mixed lymphocyte reaction (MLR) and is associated with the leukocyte activating determinants (LAD), the principal one of which in man is designated the D locus.

Because of their role as transplantation antigens, much effort has gone into attempts to assure their identity in the donor and recipient of a graft, be it a solid organ such as the kidney or tissue such as the bone marrow. Determination of the characters of these antigens is called tissue typing. The success of such efforts both in attaining good matches and in the prediction of the acceptance of the graft is limited by, on the one hand, the very extensive polymorphism of the genes and antigens, and, on the other hand, by the existence of very numerous other loci, as many as perhaps 500 in the mouse, coding for so called "minor" histocompatibility antigens, whose role in graft rejection becomes prominent when mismatch at the MHC is absent.

Research on the MHC is currently focused on three areas. Genetic studies are being carried out on recombinant strains of mice (Schreffler) to characterize the topology of the MHC and the interdependence among the various gene products in the expression of their function. For example, it has become evident that the expression of the structural gene which codes for the I-A region antigen is controlled by a gene near the I-J region (McDevitt). The appearance of the I-A incoded molecule on the cell surface, not its synthesis, requires the presence of the second gene either on the same (cis-complementation) or on the other (trans-complementation) chromosome 6.

Such studies on recombinants make use of highly inbred colonies of mice. Such colonies are also necessary for the production of highly defined mutants at the MHC and other histocompatibility loci (Melvold). Such mutants become an important resource for studies of structure-function and-antigenicity relations in the gene products when the mutations involve single-amino acid changes (Forman) or for the identification of loci when the mutations are deletions. It is with such a mutant line that a new mouse MHC locus H-2 L was identified very recently (Kohn and Melvold).

A third approach to the study of genetic regulation of the MHC employs wild mice (Klein). This permits estimation of the true degree of polymorphism of the genes as well as of heterozygosity. Thus, the finding of very high levels of hetezygosity, about 100% at the K and D loci and 80% at the I-A locus, even in inbreeding colonies in the wild strongly implies the existence of selective pressures that may well be associated with the observed correlations of MHC types with disease (see below). Thus, homozygosity might accentuate the susceptibility to a disease associated with a particular antigen which would be present in a double dose.

The second area in which much activity is evident is that of the functional roles of the histocompatibility antigens. The control mechanisms ascribed to the various loci or sub-regions of the I region of the MHC have already been discussed (David). Such a thorough dissection, analysis and correlation with function of a chromosomal region represents a most significant advance in mammalian genetics and begins to approach the level insight achieved in single celled organisms.

The genetic control of the immune response has also been investigated through immunizations with well-defined antigens in animals, where control of the response to many antigens has been found to reside in the I region, or through associations with diseases which have an immunological component with MHC antigens in man (Amos, Duquesnoy, Fudenberg, Hsia, Marsh, Stastny, and Walford). Sufficient progress has been made in determining the molecular basis for the involvement of MHC gene products in the recognition and control of the response to antigens in the mouse to permit the construction of well based hypothetical models. It appears that I-A and I-E region products must associate with the foreign antigen for it to be effectively recognized by the afferent limb of the immune system. This also seems to be the case in the recognition of certain viral antigens which must interact in some manner with the host's major transplantation antigens to elicit an immune response.

The products of other I region genes are evidently involved in recognitions among the regulatory subsets of the lymphocyte population.

The required associations of foreign antigens with MHC gene products have clear implications as to the mechanisms that underlie the observed statistical correlations between human MHC (HLA) types and various degenerative or infectious diseases. Direct investigations of these possible mechanisms are now much needed.

The third area of intense investigation of the MHC consists of physical and chemical studies of the surface antigens. Such studies employ sensitive separations of the cell surface proteins to identify the extent of the similarities and differences among the various gene products and among their various antigenic forms (Jones and McDevitt), and of determinations of exact amino acid sequence to establish evolutionary patterns (Hood), relations to other products of the immune system such as antibodies, or the chemical basis of alloantigenicity. In this connection, it might be pointed out that the carbohydrate moiety of the HLA antigens is not recognized in alloimmunizations (Strominger).

2. Transplantation

The major application of studies on the immunogenetics of man remains transplantation. The principal objective is to so reduce the antigenic disparity between donor and recipient as to minimize the tendency to reject the graft. Until recently efforts to match focused on two serologically identified loci of HLA, A and B (C is of minor importance), and at the locus identified by the mixed lymphocyte reaction (MLR), D. Matching is very clearly highly beneficial in transplants among related donors and recipients, as is matching at the D locus in renal and probably bone marrow transplantation. The inference that may be drawn is that matching a living-related donor and recipient at the two most accessible loci is effective because it also implies matching along the entire MHC and beyond. Its utility in the transplantation of organs and tissues from unrelated donors is much less clear and is the subject of continuing debate. As a result, the search continues for "the" transplantation antigen. A new candidate has appeared in the field of kidney transplantation: the product of the DR locus. This region is very closely linked to the D locus and appears to dominate the responses observed in the primed lymphocyte test (PLT) in which the proliferation of lymphocytes stimulated once by exposure to allogeneic cells is measured following a second exposure (Dausset). Matching at the DR locus has been found very promising in Europe, where matching at the A and B loci also prolongs graft survival, and is now being attempted in the U.S. The technical aspects of the procedure are difficult and a satisfactory level of consistency in DR typing is only now being approached (Third American Workshop, Fran Ward, Ed.). An extensive international study of the efficacy of DR matching in renal transplantation is being conducted under the auspices of the Eighth International Workshop on HLA Testing (Terasaki).

The limitations of the approach to matching at the MHC by typing lymphocytes must be kept clearly in mind, however. Thus, there are numerous other loci on various chromosomes that code for polymorphic cell surface structures which can, as a result, serve as transplantation antigens. The red blood cell antigens are an obvious example, but many other examples exist. The skin displays the effects of mismatching of these antigens most clearly: it is the most difficult tissue to transplant because it is at highest risk of rejection. It is in connection with these non-MHC antigens that the phenomenon of cyclic variation in the intensity of the response became apparent: alternate periods of stimulation and

suppression (Graff). This variation is undoubtedly not restricted to responses to non-MHC alloantigens. A further complication is introduced by the existence of developmental or tissue-specific alloantigens, for example endothelial and skin antigens (Bailey and Sakai). These will be missed when only lymphocytes are typed and yet will cause rejection on transplantation. Clearly, additional knowledge of these alloantigens must be sought.

Complete matching is possible only in identical twins. In all other cases, survival of the graft is totally dependent on immunosuppression of the recipient. The current mainstays of immunosuppression are adrenal cortical steroids and the cytotoxic agent azathioprine, used in rather standardized regimens. The philosophy of such treatment in renal transplantation is shifting towards minimization of drug doses with acceptance of increased risk of graft loss (Tilney). Concurrently, there is a search for more specific immunosuppressants. One such agent, anti-lymphocyte or thymocyte globulin, has been under investigation for a number of years, with variable results. The reason may well be the highly variable potency of this biological material. This difficulty is being overcome by assessing the potency of the preparations in monkeys and by carefully monitoring the lymphocyte and T cell (E-rosetting cells) levels in the recipient (F. Thomas and J. Thomas). A further advance in immunological monitoring is impending as a result of the development of reagents (sera) capable of identifying the helper, suppressor and effector T cell subpopulations in man (Schlossman). In addition to helping tailor drug doses, the sera should facilitate the elucidation of the regulatory mechanisms that determine the fate of the graft.

An apparently still more highly selective immunosuppressant coming under investigation at this time is the drug cyclosporin A. Its mechanism of action is not yet clear but appears to involve suppression of the recipient's cells stimulated specifically by the graft.

An alternative approach to immunosuppression depends on irradiation of the recipient. Studies in dogs employing supralethal irradiation followed by infusion of autologous bone marrow drawn earlier suggest that a state of high susceptibility to tolerance induction results, facilitating the acceptance of the graft (Rapaport). A similar state of susceptibility to tolerance induction follows the less drastic total lymphoid irradiation in which the marrow of the skull and long bones and the lungs are spared (Strober). This state applies not only to the recipient but also to the donor tissue in bone marrow transplantation and so avoids the development of graft-versus-host disease (GVHD). This approach clearly merits further development, and TLI is currently being attempted in renal transplantation at the University of Minnesota (Simmons).

At present, the preparation of patients for bone marrow transplantation with radiation and cytotoxic agents would be lethal were not donor marrow given. The recipient is thus at risk both from rejection of the marrow and from the development of GVHD. The two principal diseases treated with bone marrow transplantation are aplastic anemia and acute leukemia. In the former, survival rates of 85% are attained if prior blood

transfusions are avoided. The major danger is of rejection of donor cells. In acute leukemia, two year survivals of 55-60% have been achieved if the transplantation is performed prior to the end stages of the disease (E. D. Thomas). The major advances in bone marrow transplantation, other than those mentioned, have resulted from refinements in supportive care.

3. Kidney Transplantation Histocompatibility Study

The Kidney Transplantation Histocompatibility Study (KTHS) is a prospective study of the natural history renal transplantation carried out under contract by a consortium of over 40 U.S. and Canadian centers. At its inception, its principal objective was to determine the utility of matching at the A and B loci. However, comprehensive data were collected on all phases of renal transplantation, including characterizations of donors and recipients, selection procedures, intraoperative events, post operative management and outcome. A total of 2434 transplants were entered into the study in 1974-1976. Complete followup continued until the end of 1978, with some additional information still to be acquired in 1979. The data to the end of 1978 have been reviewed and corrected and are currently being analyzed by the Naval Medical Research Institute's Data Analysis Office and GTBB. The salient conclusions evident at this point, some previously known, include the following: (a) Graft survival is substantially higher when the donor is related to the recipient. This advantage depends, however, on the adequacy of matching at the A and B loci. In transplants involving related donors in which a two haplotype match is achieved, two-year graft survival is about 85%, as against about 50% when the donor is unrelated. If only one haplotype match is attained, results are much inferior though still better (about 65%) than in the latter case. Matching has no significant effect on graft survival when the donor is an unrelated cadaver. (b) The age of the recipient has an important effect on survival of kidneys from cadaveric donors, significant deterioration setting in beyond age 30, but not when the donor is living and related. (c) The effect of blood transfusions prior to transplantation is very significantly beneficial. The improvement in graft survival, evident even after very few (less than 5) transfusions is not counterbalanced by rapid broad sensitization of the recipient with consequent increased difficulty in finding a cross-match negative donor. (d) The results of transplantation even of kidneys of unrelated cadaveric donors are very satisfactory from a personal and a societal point of view. Patient survival, in contrast to graft survival of cadaveric kidneys, is over 80% at one year after implantation. Moreover, of those who retain a functioning kidney, approximately 70% are rehabilitated and return to fully normal activity. (e) Prior transplants do not reduce graft survival of unrelated cadaveric grafts.

The second phase of analysis of KTHS data which will involve in-depth evaluation of specific issues in renal transplantation will be carried out under contract by the Harvard School of Public Health.

It is also hoped that the formulation of additional multi-center trials and studies will be stimulated as a result of information generated by KTHS. One such trial, of immunological pretreatment of cadaveric kidneys

with cyclophosphamide, has been proposed, under the auspices of the American Society of Transplant Surgeons and will shortly undergo review for scientific merit.

4. HLA Serum Bank

The HLA Serum Bank is an international resource that supports all aspects of histocompatibility testing. Through a series of contracts it acquires, processes, stores and distributes human sera in bulk form and on trays. Sera are also acquired by direct purchase and by donation, the last accounting for approximately one-third of the current inventory. Bulk sera are made available predominantly for research while the principal use of the trays is in clinical applications, chiefly renal and bone marrow transplantation. Sera accepted by the bank are subjected to careful screening to characterize their behavior and validate their utility. They thus serve as standards for the research community.

During the past year, the bank distributed 13 liters of human sera in 1 ml freeze-dried samples to 150 U.S. and 94 foreign investigators. These sera were used in studies in genetics, immunology and anthropology, among others. A continuing application of great interest is study of the association of various diseases with HLA types.

The Bank has also recently acquired homozygous typing cells which will also serve as reference standards.

Approximately 35 thousand typing trays were distributed during the past year. Previously restricted to use in human transplantation, they have now also been made available for the characterization of panels of cells used to identify sera with useful specificities, as well as for selected research purposes. As a result of recent policy decisions, of the increasingly routine clinical use of sera and trays, and of our increasing understanding of where these materials can be used to greatest advantage, investigational applications will receive a higher priority and efforts will be made to have the routine clinical aspects provided for by more appropriate mechanisms. In this connection, the typing tray is being redesigned. A basic tray most directly useful in typing related graft donors and recipients and adequate in the majority of other applications will be produced and will be supported by a more limitedly available trays encompassing the rarer specificities and useful in studies of special problems and populations.

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IMMUNOBIOLOGY AND IMMUNOCHEMISTRY BRANCH

A. Scope

This Branch is concerned with the biology and chemistry of the immune system and its products. The fundamental studies which it supports on the structure and function of the immune system are directed toward acquiring a complete understanding of immune response mechanisms at their basic cellular and molecular levels as they function in health and disease.

Activities in this broadly-based program area cross traditional disciplinary lines of biology and chemistry and encompass anatomic, physiologic, pharmacologic, and microbial biology and organic, physical, and biological chemistry. Its scope includes studies of the origin, maturation, localization, and function of immunologically active cell populations, and the mechanisms involved in the induction, modulation, regulation, and expression of immune reactivity, as well as physicochemical studies of antigens and their homologous antibodies and the mechanisms and kinetics of antigen-antibody reactions.

Research activities supported within this program area are grouped in the general programs for Immunobiology and Immunochemistry and in a Program of Institute Emphasis (PIE) for Lymphocyte Biology.

B. Awards and Support Levels

Relevant activities in immunobiology and immunochemistry are supported through various mechanisms including contracts, individual post-doctoral fellowships, institutional pre- and post-doctoral training grants, career and career development awards, as well as investigator-initiated research grants.

The following shows the distribution of support by award mechanism for the activities of the Branch during FY 1979.

Immunobiology and Immunochemistry Branch
FY 1979 Awards

Award Mechanism	Number	Amount[1]
Research Grants	235	$23,733,831
Career and Career Development Awards	28	660,146
Subtotal	263	24,393,977
Fellowship Awards	19	244,446
Training Awards	9	687,721
Subtotal	28	932,167
Contracts	5	25,609
Total	296	$25,351,753

[1]Total costs, estimated using a 40% overhead for indirect costs.

The distribution of these awards by discipline is approximately 3/4 for immunobiology and 1/4 for immunochemistry. Approximately 35% of these awards were for competing new or renewal applications; the remainder represents commitments to support awards made in prior years.

Support for the Lymphocyte Biology PIE is included in the research grant category. During FY 1979, five program project awards, at a total cost of $4,280,855, were made to support this activity.

C. Program Areas and Highlights

1. Immunobiology

This program is concerned with the processes leading to immunocyte differentiation, proliferation, and production of biologically-active substances which mediate immune reactions.

Research supported within this program includes studies on the origin, maturation, and localization of immunologically active cell populations, the interactions of lymphocyte subpopulations and their interrelationships with macrophages and other leukocytes, the cellular phenomena of antigen processing, immunologic tolerance and enhancement, and the mechanisms involved in the induction, modulation, and regulation of immune responses. The cellular mechanisms involved in the induction, maintenance, expression and pathophysiology of delayed-type hypersensitive immune reactivity are also included in this category. Also relevant are studies of the lymphokines and other lymphocyte substances which activate macrophages and have pharmacologic effects on lymphocyte and other leukocytic cell functions such as chemotaxis, phagocytosis, blastogenesis, cytotoxicity, and microbial resistance.

Definition of the origin, differentiative pathways, and functional roles of the various subpopulations of lymphocytes continues to be a focus of considerable investigative effort. Although it is recognized that one population of mature lymphocytes (T) is thymus dependent and another population (B) is thymus independent, their progenitors and their mechanisms of functional maturation are still not precisely identified. Studies on the ontogeny of lymphocytes being conducted by Irving Goldschneider (AI 09649 and AI 14743, University of Connecticut Health Center) are centered on the identification and isolation of lymphohemopoietic stem and progenitor cells from rat bone marrow. Using specific antilymphocyte sera and the fluorescence-activated cell sorter, he has enriched preparations of pluripotent hemopoietic stem cells and thymocyte progenitors and has identified two subsets of B lymphocytes which represent sequential stages in B cell development. He has demonstrated that the Thy-1 antigen, and T cell surface molecule, is present on the least mature members of the B cell series but is absent on mature B cells; for this reason, the Thy-1 antigen can serve as a useful marker of the early stages of B cell development. He also has shown that a subset of bone marrow cells which contain the enzyme terminal deoxynucleotidyl transferase (TdT) is composed almost exclusively of thymocyte progenitors. Other data indicate that nucleotide metabolizing enzymes

such as adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) play important roles at different stages of T lymphocyte development. Of interest are the results of related studies in man by Rochelle Hirschhorn (AI 10343, New York University) who has found that approximately one-half of the patients with severe combined immunodeficiency disease are deficient in ADA and PNP. She believes that these enzymes are crucial for normal differentiation and function of immunocompetent cells and suggests that the pathophysiology of this disease involves a primary inhibition of T cell maturation and a toxic effect on cells differentiating after antigenic stimulation. She has found that some of the immunodeficient and enzyme deficient patients can be immunologically reconstituted by infusion of irradiated erythrocytes which contain ADA and has proposed that deoxyadenosine triphosphate is the mediator of the toxic effect in ADA deficiency. Defects in human B lymphocyte development also have clinical expressions. Alexander R. Lawton, III (AI 11502, University of Alabama in Birmingham) has found that a defect in differentiation of B lymphocytes from their pre-B cell progenitors occurs in one form of congenital agammaglobulinemia, while failure of pre-B cell development occurs in a type of hypogammaglobulinemia associated with a tumor of the thymus gland.

The results of other ontogenic studies similarly have led to recognition of new developmental markers. On immunization of mice with teratocarcinoma cells, Barbara Knowles, recipient of a Career Development Award (AI 00053, Wistar Institute), has obtained an antibody which reacts with embryonic carcinoma cells of both mouse and human origin and with some preimplantation stage mouse embryos. She believes that this stage-specific antigen on the cell surface is a hallmark at the undifferentiated state; it is first detected on cells of the 8-cell stage embryos and is lost by embryonic carcinoma cells when they differentiate. Using a histochemical stain for viable DNA, Michael R. Loken (AI 14872, University of Chicago) has developed a method to quantitatively discriminate between viable B and T lymphocytes in both mouse and man. He believes that this histochemical stain will serve as an important marker to differentiate lymphocyte subpopulations based on their cytochemical composition rather than on their cell surface molecules. The results of preliminary studies of mouse bone marrow cells suggest that differentiating lymphocytes can be discriminated on this basis.

Although the functional distinction of lymphocyte populations is convincingly documented, the purpose and mechanisms of their interactions with each other and with macrophages is still being examined. It is clear that B lymphocytes differentiate to become antibody-secreting plasma cells and that T lymphocyte subsets can exert helper or suppressor effects on B lymphocytes as well as effector functions in transplantation reactions and in cell-mediated immune reactions to various microbial agents. The cooperative and effector functions of T lymphocytes have been found to correlate well with the presence of a cell surface molecule, the Ly antigen. It also is known that these functions are controlled and regulated by other cell surface antigens which are products of the genes in the major histocompatibility complex (MHC). The regulatory role of MHC products and the functional role of other cell surface antigens in the immune response have been defined most extensively in the mouse although systems analogous to

the H-2 component of the murine MHC have been identified in other animals and in man.

On recognition of the regulatory role which the MHC exerts on the immune system, efforts to clarify the responsible mechanisms have intensified. To facilitate research in this area, the program has contractually supported the acquisition and distribution of antisera with specificities against various mouse cell surface antigens. A supply of antiserum against many of the H-2 gene products has been available and antisera specific for antigens of the I region of the H-2 complex have recently been prepared by Donald C. Shreffler (AI 6-2502, Washington University) for distribution to investigators.

Evidence obtained in several experimental systems has convincingly demonstrated that efficient physiological interactions among macrophages, T cells, and B cells require that these cells share membrane molecules encoded for by the major histocompatibility complex of the species. David H. Katz (AI 13874, Scripps Clinic and Research Foundation) has provided substantial evidence that the genes controlling interactions between T and B lymphocytes are located in the I region of the mouse H-2 complex. He has found that T lymphocytes will not exert effective helper functions for B cells when these cells differ at the relevant I region locus. He believes that self-recognition is the critical mechanism by which cell-cell communication takes place in the immune system. In a related project (AI 13781), he has obtained evidence indicating that the process of differentiation of stem cells and their progeny also may be critically regulated by MHC gene products. Using bone marrow chimeras, he has demonstrated that lymphocyte differentiation may be "adaptive" to the environment in which it takes place. He believes such an "adaptive differentiation" results in preferential interactions among cells that have undergone their differentiative processes in the same genotypic environment; "adaptive differentiation" thus may represent a manipulative approach to define genetic and molecular mechanisms responsible for cell-cell interactions.

Genetic restrictions imposed by products of the I region of the H-2 complex on interactions between macrophages and immune T cells are being investigated by Carl W. Pierce (AI 13915, Jewish Hospital of St. Louis). Using antigen pulsed macrophages, he has found that naive lymphocytes will respond to antigen even if it is presented on an histoincompatibile macrophage. However, the secondary immune response is genetically restricted; the immune T cell must be stimulated with antigen-bearing macrophages which are genetically identical to the macrophage that presented the antigen during the primary immunization. He has demonstrated that such genetic restrictions are controlled by products of the I-A subregion of the H-2 complex and believes that these restrictions involve active suppressive phenomena when antigen is presented on an inappropriate macrophage. In a related study, Judith A. Kapp-Pierce (AI 13987, Jewish Hospital of St. Louis) has found that an antigenspecific suppressor T cell factor is activated when nonresponder lymphocytes are stimulated by antigen; suppression by this factor is mediated by a molecule encoded by the I-J subregion of the H-2 complex. She also has shown that this suppressor factor does not require participation of macrophages for effective suppression; she

suggests that the suppressor factor acts directly on primed B cells. The results of similar studies by Donal B. Murphy (AI 14349, Yale University) suggest that the different functional subpopulations of immunocompetent lymphocytes express different products of the I region on their surface. He has demonstrated that suppressor T cells bear an I region antigen not found on suppressor T lymphocytes. Of interest is his observation that, although different T cell subpopulations bear distinct I region products, all or many of these products appear to be expressed on the surface of the macrophage so that its interaction with these lymphocytes would not be encumbered by this type of genetic restriction.

The mechanisms by which antigens are presented to T cells by macrophages to initiate the immune response are being examined by David W. Thomas (AI 14226, Jewish Hospital of St. Louis). He is using macrophages modified with trinitrophenyl (TNP) as a model to study the "immunologically relevant" processing of antigen by macrophages which is necessary for its subsequent recognition by T cells and which is different from the degradative and metabolic breakdown of antigen by macrophages. His results indicate that T cell recognition of TNP modified macrophages does not simply involve a surface display of TNP but that macrophages somehow process TNP to create an efficient immunogen. He has concluded that T cells recognize and respond to macrophage-bound antigen only when it is associated with surface products of the I region of the MHC. His physiocochemical studies, however, have provided evidence that TNP is not directly conjugated to macrophage I region products. On this basis, he believes that antigen processing by macrophages involves a physical association of antigen with I region products rather than an alteration of "self" by chemical linkage of antigen with I region products.

Although it is recognized that physical contact between macrophages and lymphocytes is necessary in the immune response, there is considerable evidence that each of these cell types produce factors which also are involved in the initiation of the immune response. Byron H. Waksman (AI 06455, Yale University), for example, has demonstrated that the lymphocyte activating factor which stimulates helper T cells is a macrophage enzyme. He has provided evidence to indicate that this enzyme alters lymphocyte surfaces so that calcium incorporation is stimulated and cyclic nucleotide activity is consequently enhanced. In a reciprocal fashion, Michael P. Rabinovitch (AI 10969, New York University), has demonstrated that interferon, an antiviral agent produced by lymphocytes, controls the phagocytic functions of macrophages. He has demonstrated that macrophages obtained from animals treated with interferon inducers exhibit enhanced phagocytosis while macrophages obtained from mice treated with an antibody which neutralized interferon had diminished phagocytic activity. Interferon also has been shown to have immunoregulatory effects on lymphocytes; Barry R. Bloom (AI 09807, Albert Einstein College of Medicine) has found that interferon was produced and generated suppressor T cells when lymphocytes of normal individuals were cultured with measles virus. Of interest is his observation that lymphocytes from patients with multiple sclerosis failed to produce interferon when similarly exposed to measles virus. He believes this selective defect in response to measles virus in patients with

multiple sclerosis may play a significant role in the persistence of this disease.

Cell-cell interactions are required not only to initiate the immune response but also to regulate it. It is now recognized that the help and suppression provided by T cell subsets is carefully balanced within the responding host. As demonstrated by Richard K. Gershon (AI 10497, Yale University), helper T cells, on stimulating B cells to produce antibody, also become susceptible to regulation through a feedback inhibition mechanism by inducing suppressor T cells. In collaboration with Harvey Cantor (AI 13600, Sidney Farber Cancer Institute), the functional subsets have been identified on the basis of differentiation molecules, termed Ly antigens, present on their surfaces. Thus, helper cells have been shown to be Ly 1+ while suppressor cells are Ly 2, 3+. Cantor recently has provided convincing evidence that the Ly 1 helper cell can induce an uncommitted Ly 1, 2, 3+ subset to participate in specific suppressor activity. In this context, Gershon believes from his current studies on the cellular feedback inhibition mechanism that suppressor dysfunction is a cause of at least some autoimmune diseases. He acknowledges, however, that, in some autoimmune diseases, the aberrant activity of suppressor T cell is not due to inherent dysfunction of that cell but due to defects in other cells that regulate it or are regulated by it.

Although research on functional subsets of T lymphocytes is proceeding at an accelerated pace, it is recognized that this work is technically limited by the need to separate the subsets in adequate numbers for study. This difficulty may be obviated by the recent work of Frank W. Fitch (AI 04197, University of Chicago) who has developed a method to isolate and culture cloned lines of reactive T cells, including separate lines with distinct helper and suppressor activities. In a similar fashion, research in this area should be facilitated with the Program's acquisition of a supply of antisera against Ly antigens which now are available for distribution to qualified investigators. These sera were prepared under contract through the efforts of Jeffrey A. Frelinger (AI 7-2528, University of Southern California). Development of methods and materials for preparing antisera against newly-recognized Ly antigens and other immunologically-relevant cell surface molecules also is being supported through a contract with Edward A. Boyse (AI 8-2541, Sloan Kettering Institute for Cancer Research).

Approaches to obtain helper and suppressor factors from T cell subsets also are being explored. Sirkka K. Kontiainen (AI 13145, University of Helsinki) has developed methods to obtain active suppressor factor from cultured mouse suppressor T cells. Using anti-Ly antiserum, she has demonstrated that the target for suppression is the Ly 1+ helper T cell and has characterized the suppressor factor as having both an antigen combining site and a determinant encoded by the I region of the H-2 complex. Using a similar approach, Marc Feldmann (AI 15653, University College, London) has obtained a helper factor from cultured human peripheral blood lymphocytes and has developed an assay for its activity using mouse spleen cells. This technology will permit analysis of human lymphocyte immunoregulatory

function in disease states and should facilitate attempts to selectively manipulate the immune response in immunologically impaired individuals.

In addition to the factors produced by T cells which have immunoregulatory functions, another group of lymphocytes products, termed lymphokines, is receiving considerable investigative attention. Lymphokines have been recognized as being the mediators of a variety of immunologic reactions including inflammation, graft rejection, tumor and other cell killing, and delayed hypersensitive reactions. The cellular and molecular mechanisms of action of lymphotoxin, a cell lytic effector, are being investigated by Gale A. Granger (AI 09460, University of California at Irvine). He recently has found that human lymphotoxin molecules form an interrelated system of cell toxins which is composed of noncovalently associated subunits. He has identified three separate types of molecules in this system; it contains lytic subunits, condensing molecules which facilitate association of the subunits, and antigen binding receptors. He has found all three types of molecules are released predominantly from T lymphocytes and that the lytic action of the molecular complex is directed by the component containing the antigen binding receptors. In similar studies of another human lymphokine, macrophage migration inhibition factor, Stanley Cohen (AI 13258, University of Connecticut Health Center) has obtained evidence that it also may consist of subunits, noncovalently associated into an active complex. In a related study (AI 12477), he is exploring ways to therapeutically alter lymphokine-mediated reactions in the intact host. He has found that various monosaccharides which are capable of inhibiting lymphokine activity in vitro also are effective in suppressing in vivo manifestations of cell-mediated immunity. For example, L-fucose was demonstrated to inhibit the delayed hypersensitive skin reaction induced by antigen in actively immunized guinea pigs. Furthermore, he and Takeshi Yoshida, recipient of a Career Development Award (AI 00082, University of Connecticut Health Center), have been able to induce mediator production in vivo and abolish delayed hypersensitive skin reactivity in actively immunized guinea pigs. They found that desensitization could be passivley transferred using mediator-containing serum from desensitized animals; the results of physicochemical studies of such sera excluded the participation of antibodies or immune complexes and suggested that the desensitizing substance may represent another type of endogenous lymphokine.

2. Immunochemistry

Research grouped in this program category centers on the defined molecular components of the immune system and utilizes chemical and physicochemical approaches for study of immunologic reactants and reactions. Studies on the chemical composition and structure of humoral and secretory immunoglobulins and of natural and synthetic antigens, as well as of the mechanisms and kinetics of antigen-antibody reactions and of the mechanisms of immunoglobulin biosynthesis and its regulation, are included within this program area. Also relevant are studies at the molecular level of cell surface components related to immunologic activity and of subsurface cytoskeletal and contractile assemblies believed to play a role in cellular activation. In addition, the newly-developing field of immunopharmacology,

which is concerned with the identification, characterization, isolation, or synthesis of chemical regulators of immune function and the elucidation of their mechanisms of action is supported in this program area.

A major aim of studies supported by this program is to elucidate the primary structure of antibodies and to correlate their structure with their antigen binding, specificity, and other biological functions. The results of structural studies have established that an immunoglobulin basically consists of two identical light (L) and two identical heavy (H) chains, linked by disulfide bridges to form a two-fold symmetrical molecule, each side of which consists of an L and an H chain. Two clases of L chains (kappa and lambda) and five H chains (gamma, alpha, mu, episolon and delta), which determine the immunoglobulin class (IgG, IgA, IgM, IgE, and IgD, respectively) have been recognized. Limited proteolysis of a typical IgG molecule results in three fragments of nearly equal size, 2 Fab fragments and an Fc piece. It is now clear that the Fab components contain the antigen binding (combining) site and that the Fc fragment plays an important role in complement fixation. Amino acid sequence studies of immunoglobulins have demonstrated the existence of three domains with constant sequences on the heavy chain (C_H) and one domain of variable sequence (V_H); each light chain consists of two domains, a constant region (C_L) and a variable domain (V_L). The close association of the V_L and V_H domains in the Fab fragment, containing the combining site, produces a continuous hypervariable surface which can be readily altered by amino acid substitution, insertions or deletions, and provides an extremely large number of structural combinations which are believed to confer immunologic specificity.

Considerable effort now is being expended to gain an understanding of the mechanisms controlling and regulating immunoglobulin biosynthesis and of the basis for antibody diversity. Attempts to explain the diversity of variable region sequences have centered on a multiple germ-line gene theory which presupposes that all information necessary for antibody production is encoded in the germ line and is inherited or on a theory of somatic diversification which assumes that a limited number of germ-line genes are diversified during an individual's lifetime by somatic processes, such as point mutations, recombinations or introduction of errors into the gene sequence, followed by repair to yield a new set of encoding genes.

Amino acid sequence analysis and structural studies provide one approach to understanding immunoglobulin diversity. The results of studies by Fred Karush (AI 09492, University of Pennsylvania) support the view that the V_H genes expressed in murine IgG antibody are encoded in the germ line. A contract project under the direction of Elvin A. Kabat (AI 8-2158, Columbia University) in collaboration with Tai T. Wu, recipient of a Career Development Award (AI 70497, Northwestern University), supports the tabulation and analysis of immunoglobulin sequence data reported in the world's scientific literature. Using the PROPHET Computer System, this project provides the concerned scientific community with a unique and invaluable data resource and serves as a source of data for molecular model building and statistical predictions of immunoglobulin molecular structures. The accumulated data base on immunoglobulin variable region sequences has been

published and a series of computer-assisted analyses of the data base has been completed. Evidence has been obtained to suggest that the amino acid sequence characteristic of segments in the variable region of an immunoglobulin is uniquely associated with antibody specificity. Computer-generated inferrences concerning amino acids at key positions in light and heavy chains agreed well with structural conclusions obtained by X-ray crystallography. Additional predictions of the three dimensional structure of the combining site have led to the suggestion that the structural gene for the variable region of an antibody may be assembled from "mini genes" which have been conserved in the germ-line. Support for the germline theory also has been provided from studies conducted by Joseph M. Davie (AI 11635, Washington University) who interprets the uniformity of isoelectric focusing patterns of rat antibodies as being consistent with the view that these antibodies are products of germ-line genes.

In contrast, using antibodies prepared against the combining site, or idiotype, of an immunoglobulin, Alfred Nisonoff (AI 12895, Brandeis University) believes that it is not necessary for a mouse to inherit V_I structural genes. He feels that antibody diversity can be generated through a random process of somatic mutation, rather than through a programmed, genetically-controlled series of mutations. The results of his related studies (AI 12907 and AI 12908) are providing strong evidence for the importance of an idiotype-antiidiotype network as a mechanism regulating the immune response. He believes that the idiotypespecific regulation by antiidiotype antibody and by suppressor T cells is an important physiological mechanism determining the magnitude of a given immune response. The results of research by Matthew D. Scharff (AI 05231, Albert Einstein College of Medicine) and Malcolm L. Gefter (AI 13357, Massachusetts Institute of Technology) support the view that immunoglobulin diversity is generated by somatic diversification. Studies of immunoglobulin synthesis at the level of the nucleus of a cell also has served as an approach to evaluate theories of immunoglobulin diversity. The results of sequence studies of DNA and messenger RNA and of their function in immunoglobulin synthesis conducted by several investigators including Ursula B. Storb (AI 10685, University of Washington), Randolph Wall (AI 13410, University of California at Los Angeles), and Janet M. Stavnezer (14617, Sloan-Kettering Institute for Cancer Research) are difficult to reconcile with a strict germ-line theory and argue for a somatic process of immunoglobulin diversification.

Efforts are now being made to extend such studies to man although they are still in their preliminary stages. Moyra Smith-Wright (AI 14287, Mt. Sinai School of Medicine), using a somatic cell hybridization technique, has demonstrated that the structural genes encoding IgG heavy chains are located on human chromosome 6. Using a serologic approach to examine the diversity and inheritance of immunoglobulin genes, An Chuan Wang (AI 13388, Medical University of South Carolina) has discovered a new genetic marker which is located in the V_H region of human immunoglobulins G, M, and A. This observation represents the first description of a human immunoglobulin variable-region genetic marker; the results of pedigree and population analyses suggests that it has a dominant mode of inheritance.

Although sequence and serologic studies of serum immunoglobulins are not generally restricted by the availability of material for study, similar studies of cell-bound immunoglobulins and other cell surface receptor molecules have indeed been limited by the small amounts of material available for study. Recent technical advances may obviate this problem, however. Gerald M. Edelman (AI 09273, Rockefeller University) had developed a system to synthesize authentic mouse β_2-microglobulin and its precursor in rabbit reticulocytes using messenger RNA from murine tumor cells; its authenticity has been verified by radiochemical-sequence analysis. The same system is now being used to synthesize H-2 antigens, TL antigens, and other cell surface proteins found on lymphoid cells. Similarly, Sheldon Dray (AI 04073, University of Illinois Medical Center) has been employing liposomes, synthetic micellar structures which mimic cell surface lipid structures, to selectively insert functional messenger RNA into cultured cells. He recently has obtained evidence which indicates that cultured human epithelial carcinoma cells treated with liposomally-encapsulated rabbit globin messenger RNA are stimulated to produce a globlin-like protein. Matthew D Scharff (AI 05231, Albert Einstein College of Medicine) is employing somatic cell genetic technology to refine the Kohler-Milstein technique for production of hybridomas, the product of fusing malignant plasma cells and normal antibody-forming cells. He has developed an extensive library of drug-marked myeloma cells for use in fusion experiments and has adapted the fusion technology to significantly improve the frequency of functional hybridomas. Further application of the hybridoma technology, as well as the insertion and translation of messenger RNA in cultured cells, has the potential to provide an unlimited source of homogeneous antibody and other immunologically-important molecules for study. Application of recombinant DNA technology to this problem can similarly be expected to expand the supply of study material. Randolph Wall (AI 13410, University of California at Los Angeles) already has successfully inserted mouse myeloma immunoglobulin DNA into a bacterial plasmid and has isolated recombinant clones which contained all of the light chain constant and variable region coding sequences.

Efforts to define and understand the molecular basis of antigenicity and of the antigenic or determinant site similarly has progressed by application of amino acid sequence analysis. For example, M. Zouhair Atassi (AI 13181, Mayo Foundation) and Ahmed F. Habeeb (AI 14791, University of Puerto Rico) have employed hen egg white lysozyme as a model antigen and have systematically sequenced it. They have identified three peptide fragments which contain the antigenic sites of this protein. Using a method of surface simulation, they have synthesized peptides containing the component amino acid residues in appropriate structural order and have been able to accurately define the voundary, residues, and conformational structure of the three determinants of the native protein. Their results clearly demonstrated that these three sites quantitatively accounted for the total antigenic reactivity of the native protein. Thus, the entire antigenic structure of lysozyme has been precisely defined. In a related study, Eli E. Sercarz (AI 11183, University of California at Los Angeles has demonstrated that peptide fragments containing these determinants can be distinguished by their different immunologic functions. The L_1 peptide, containing amino acid residues 1-12, induced a suppressor cell response;

the L_2 peptide containing amino acids 13-105 induced helper cells while the L_3 peptide had no effect. This demonstration of helper and suppressor sites on the same molecule provides a powerful tool to investigate the cellular mechanisms of immune recognition.

A somewhat different approach to the problem of antigenicity is being employed by Conrad Scheurch (AI 12509, State University of New York at Syracuse) who has prepared a variety of synthetic polysaccharides, modeled after the naturally occurring bacterial polysaccharide dextran as well as yeast and fungal mannans. In addition to providing synthetic antigens with well defined chemical structure for immunologic study, this work is purposely designed to identify clinically useful plasma expanders, like dextran, which would be safer for use because they do not possess dextran's allergenic properties. These synthetic polysaccharides are now being examined for allergenic potential.

3. Program of Institute Emphasis for Lymphocyte Biology

This Program currently supports five program projects, each of which is a multidisciplinary research effort integrating the technical expertise of cell biologists, geneticists, cellular immunologists and immunochemists and is directed by an acknowledged leader in the field. Their combined studies are designed to expand knowledge of the morphologic and functional heterogeneity of lymphocyte populations and to develop the capability for identification and selection of lymphocyte subpopulations with specific immune reactivity or antigenic composition, for hybridization of such populations and for selective production of specific lymphocyte products. The objective of this Program is to acquire as complete a knowledge as possible of the life history of immunocompetent cells and of the physiologic and external factors that determine their fate and function in vivo and in vitro.

This program was initiated approximately seven years ago with the award of four program project grants, each directed toward the goal of understanding the biology of the lymphocyte and having different but complementary approaches. Three of the original projects have successfully undergone competitive peer review and have received renewal of support for a five-year period. In addition, support for two new program projects has recently been provided, one each in FY 1978 and FY 1979.

The program project directed by Matthew D. Scharff (AI 10702, Albert Einstein College of Medicine) has focused its approaches on the molecular biology and somatic cell genetics of the immune system to define the genetic and molecular control mechanisms of immunoglobulin synthesis, the structure and synthesis of the antigens of the major histocompatability complex (MHC), and the functions and interactions of T and B lymphocyte populations and macrophages. In many of these studies, continuous cloned cell lines and variants derived from these cell lines are being used to study the role of the cells and their products in the immune response. For example, Dr. Scharff and his associates have devoted considerable time and effort to generate immunologically functional hybrids between mouse myeloma cells and spleen cells from immunized mice. They have developed a technology

for fusing meyloma and spleen cells at a high frequency and some of the drug marked cells which are now widely used to prepare hybridomas orignated in their laboratory. They have provided cell lines and assistance to well over a hundred investigators around the world to establish this technology in their own laboratories. Studies within the program project have been productive and important contributions have been made. Cultured mouse myeloma cell lines have been used to examine the genetic control of the expression of immunoglobulin genes. Analysis of antigen-binding variants of these cell lines indicate that the variants have small structural changes in the V_H region, arise spontaneously at a very high frequency, and may be related to the normal process which regulates the generation of antibody diversity. Primary sequence analysis of constant region variants has established that they arise as a result of crossing over between two closely related constant region genes. Studies of the biochemical properties and primary structure of the H-2 MHC prodcuts in an attempt to relate functional and genetic properties with molecular structure have suggested that discrete amino acid changes in the H-2 glycoproteins are directly related to their immunologic specificity. In order to pursue the mechanisms underlying the complex and coordinated functions of macrophages and to identify the molecular basis for modulation of these functions by products of activated lymphocytes, physiologic and functional studies of cloned macrophage-like cell lines have been initiated. An unusual macrophage-like cell line has been established which can migrate, suppress antibody production by hybridoma cell lines and suppress mitogenic responses of T and B cells. These differentiated functions have not been previously observed in continuous cell lines. Insulin was found to depress phagocytosis in these cells but this effect could be overcome by cyclic nucleotides, establishing for the first time that cyclic nucleotides play an important regulatory role on the phagocytic process. The functional status of immunoregulatory mechanisms in chronic infections also is being investigated. When stimulated with specific antigen, T lymphocytes from patients with lepromatous leprosy have been found to suppress mitogenic responses; this observation may explain the anergic state frequently seen in these patients. In contrast, patients with multiple sclerosis exhibit defective T suppressor and cytotoxic functions which may contribute to the persistence of the disease.

The approach directed by Gerald M. Edelman (AI 11378, Rockefeller University) centers on the analysis of molecules and molecular assemblies involved in the control and recognition of antigenic stimuli at the surface and within the cells of the immune system. The results of his studies have provided several insights into the nature of recognition and control events in lymphocyte function. Of special interest is his evidence for the existence of a surface modulating assembly (SMA) which regulates cell surface receptors and mediates transmembrane signalling for functional cellular responses. Dr. Edelman and his associates picture the SMA as a three-component system consisting of cell surface receptors that penetrate the cell membrane, submembranous microfilaments which coordinate receptor movement, and microtubules which provide anchorage for the receptors and propogate signals to and from the cell surface. To study the mobility of the cell surface receptors and the molecular basis for transmembrane control of receptor mobility, the fluorescence photobleaching recovery

method has been adapted to directly measure surface receptor mobility on lymphocytes. Using this technique, different classes of receptors have been found to be under separate control mechanisms. A viral gene protein has been established as a valid probe to dissect the stimulatory sequence leading from the cell membrane to the cytoplasm and the nucleus and to study the enzymes involved in cellular DNA replication. Evidence has been obtained which indicates that the microtubules and microfilaments which form the cytoskeleton act as signal regulators in controlling cell growth and division. Lectins also are being employed to probe the mechanisms of mitogenesis; favin, like concanavalin A, is mitogenic but has been found to have a subunit structure very different from that of concanavalin A and, consequently, a different capacity to bind cell surface glycoprotein receptors. Favin thus offers an alternative probe for examining cell surface events. The results of structural studies of cell surface glycoproteins provide evidence that these surface receptors from aggregates with each other and with antigenic components through formation of a transient disulfide bond. To further understand the nature of molecular interactions at the cell surface, X-ray crystallography is being applied to study the structural organization of mitogenic stimuli, particularly concanavalin A.

Evidence has been obtained to suggest that the normal geometry of concanavalin A is dramatically altered in the presence of calcium and manganese ions. These changes in structural geometry are associated with mitogenic activity and are believed to be involved in the binding of concavalin A to the carbohydrate components of surface glycoproteins. Although Dr. Edelman's research is centered on the role of the SMA in the immune response, his results suggest that the SMA has a key role in controlling cell movement, growth, and differentiation in general.

The program project directed by Jonathan W. Uhr (AI 11851, University of Texas Health Science Center at Dallas) is centered on the study of the biochemistry and biology of lymphocyte surface molecules. The structure of immunoglobulins on the membrane of B cells is being studied in order to define its attachment to the plasma membrane and to clarify the molecular interactions that occur after surface immunoglobulins react with specific antigens. The broader purpose of such studies is to obtain an understanding of the molecular mechanisms which underlie signalling of B lymphocytes and control their differentiation. Studies of T cells emphasize the process of immune recognition; efforts are being made to biochemically characterize the T cell receptor and to define the role of Ia antigens in the interaction of antigen-pulsed macrophages with T cells. Relevant studies include the role of major histocompatibility complex products in recognition of viral infected cells and structural analysis of these products to develop insights into their evolution genetic control and function. The major concern and principal contributions of Dr. Uhr's project have been in the area of the ontogeny, regulation, and function of B cell surface immunoglobulins. An immunoglobulin D (IgD)-like molecule was found on the surface of circulating murine lymphocytes; ontogenic studies showed that it appeared after immunoglobulin M (IgM) when mice were approximately one week of age. The results of additional studies have led to the acceptance of a model which assigns an important role for IgD in the development and function of B cells. In this model, immature B cells first

develop surface IgM, then acquire surface IgD to become "double bearers"; some cells subsequently lose surface IgM to become cells that bear only surface IgD. Dr. Uhr and his associates have demonstrated that cells which bear only surface IgM become tolerized after encounter with antigens. They also have shown that surface IgD acts as a triggering receptor so that cells that bear both surface IgM and IgD give a primary IgM response after encounter with antigen; cells that bear only surface IgD give an IgG response after encounter with antigen. On stimulation with lipopolysaccharide, cells with surface IgM enlarge and produce a polyclonal IgM response; cells with surface IgD do not exhibit an IgM polyclonal response but instead undergo blastogenesis and proliferation. Similar results were obtained using antigenic stimulation. It was concluded from such studies that cells bearing surface IgD are the major progenitors of IgG antibody forming cells in the secondary antibody response. Long-term memory cells bear small amounts of both surface IgM and IgG. Studies of surface receptors on T cells have similarly led to important conceptual advances. Evidence has been obtained to indicate that specific binding of antigens coupled to autologous macrophages by T cells represents the initial event in immune stimulation. The macrophage plays a key role in this stage because T cell receptors do not specifically bind to native exogenous antigens. Structural analyses of T cell surface receptors have demonstrated a marked homology between the products of the major histocompatibility complex of humans, mice, and guinea pigs, implying analogies in function.

The program project directed by Baruj Benacerraf (AI 14732, Harvard Medical School) is designed to gain understanding of the genetic controls of the immune system and of the regulation of functional expressions of immunocompetent cells. Component projects are concerned with the identification and function of immune response genes, the induction, regulation, and function of helper and suppressor T cells, and the identification and function of surface receptors on T and B cells. Using genetically-determined responder and nonresponder strains of mice, Dr. Benacerraf and his associates have found that antigen-specific suppressor factor derived from T cells nonresponder mice. They also have found that specific suppression can be eliminated by administration of antisera specific for I-J gene-controlled determinants; immunopotentiation of nonresponder mice with this antiserum was demonstrated to reflect a reduction of specific suppressor responses. In other studies, macrophages of responder mice were shown to be able to present antigen in an immunogenic form and to play a central role in regulating the balance of activated helper and suppressor T cells; these findings suggested that suggested that an Ir gene defect at the macrophage level any account for the predominant suppressor T cell responses in nonresponder mice. The studies of murine B cells have led to the detection of monovalent and divalent IgD on the cell surface; evidence has been obtained to suggest that both forms of IgD are native to the cell surface and may represent different forms of attachment of these molecules to the membrane of the cell. A detailed analysis of the aggregation or "capping" of cell surface molecules has led to the demonstration of two functionally different mechanisms. The first type which occurs spontaneously appears to be an active process involving a direct link between the surface molecules and the cytoplasmic contractile apparatus.

This type appears to be related to the process of locomotion; myosin was found to be concentrated in the cytoplasm directly beneath the capped area. The other type occurred when B cells were treated with antigens, antibodies against cell surface receptors, or mitogens and appeared to result simply from aggregation of crosslinked molecules in the plane of the membrane; this type of capping did not result in a redistribution of cytoplasmic myosin or stimulation of cell locomotion. It is implied that the interaction of surface immunoglobulins, at the molecular level, with other components of the cell membrane and cytoplasm represents the earliest step of specific antigen recognition and subsequent triggering of B cells. In other studies, the IgG-bearing B cell subpopulation from human spleen has been isolated and characterized; these cells have IgM or IgD on their surface and give rise to nearly all of the IgG- and a significant franction of the IgM-secreting cells after stimulation by mitogen. The recognition of an antigenic determinant found both on murine macrophages and on human monocytes using hybridoma antibody has made it possible to use the fluorescence-activated cell sorter to prepare pure populations of human monocytes from peripheral blood for subsequent functional studies. Also, by using a cytofluorometric technique, an approach has been developed to recognize small numbers of malignant B lymphocytes in a population of normal lymphocytes. This method may facilitate the planning and monitoring of therapy in patients with lymphoid malignancies.

The program project directed by Dr. Carl W. Pierce (AI 15353, Jewish Hospital of St. Louis) was awarded in FY 1979 and has the immunobiology of the major histocompatibility gene complex as its central theme. Dr. Pierce is an outstanding cellular immunologist who has made important contributions to this area; in recognition of his accomplishments, he was the 1979 recipient of the Parke-Davis award presented by the American Society for Experimental Pathology. His co-principal investigators at Washington University, Dr. Donald Shreffler and Dr. Joseph Davie, are equally outstanding in their fields. Their combined efforts represent a multidisciplinary approach to several problems of fundamental importance to an understanding of the mechanisms and functions of the major histocompatability complex. Areas to be examined include the nature of the interactions between histocompatability gene products and conventional antigens in the generation of effector lymphocytes, the structure of genetically restricted suppressor factors, the structure and genetic relationships of components of the complement system that are produced by genes of the major histocompatability complex, the identification and structure of gene products on T cells and macrophages, and the identification and characterization of receptor molecules on the surface of T lymphocytes.

This Program of Institute Emphasis represents a major investment of the IAIDP and NIAID in the concept that advances in the understanding of the behavior of lymphocytes can be effectively achieved through carefully organized efforts by large, highly interactive groups led by well-established and highly productive senior scientists. To date, there is every indication that this program has been successful and productive and that it will continue so in the future.

MICROBIOLOGY AND INFECTIOUS DISEASES PROGRAM

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MICROBIOLOGY AND INFECTIOUS DISEASES PROGRAM

Annual Report, 1978-1979

Director's Report

The accomplishments of the five branches of the Microbiology and Infectious Diseases Program (MIDP) are summarized in the following sections; activities in international health are reviewed at the end of this section. This report addresses the implications of these accomplishments and activities for the future, with particular emphasis on virology.

Humans are infected by an astounding number and variety of organisms, ranging in size and complexity from tiny viruses which are little more than nucleic acids to large multi-cellular parasites. These infectious agents and their progeny have been living together with man and his progeny for millenia, and both have changed as a result. Concurrently, the physical and socio-economic environments shared by man and his microbial companions have changed markedly, at least in some parts of the world, and chemicals have been developed which may be introduced into man or his environment to inhibit the growth of microbes and parasites or to limit their transmission. And, in the last two hundred years, man has tamed certain of these agents, or their toxins, so as to produce attenuated vaccines (smallpox, yellow fever, poliomyelitis, measles, mumps, rubella) or toxoids (diphtheria, tetanus) which, if properly used, provide virtually complete protection against "wild" or natural strains. The net result of all this has been that, in the developed world, many serious life-threatening infectious diseases, particularly those of childhood, have been prevented with a consequent increase in life expectancy. A major victory for man!

But this is no time to be complacent. Infectious diseases are still responsible for nearly 25% of all visits to physicians in the U.S. each year, and they account for 65% of all such visits for children under 16 years. In some developing countries, up to 40% of children still die before the age of five years because of infectious diseases. Microbes and their vectors have fought back around the world. Malarial parasites have become resistant to once curative drugs; mosquitoes which transmit encephalitis viruses and malarial parasites have become resistant to insecticides; bacteria have developed resistance to antibiotics and the ability to transfer this resistance to other bacteria. A potential victory for microbes!

Bacteriology

It is appropriate, then, that NIAID encourage additional studies on mechanisms of antibiotic resistance. In doing so it will not only improve the care of patients with bacterial infections, but it will also contribute basic information regarding man and microbes, for current knowledge of molecular genetics derives largely from studies of bacteria and viruses.

One of the major recent discoveries in this field is that a number of specific DNA sequences existing at multiple locations on prokaryotic genomes can transpose and translocate spontaneously to new sites by a mechanism that is independent of the host's normal recombination system. Progress in the identification, mapping and characterization of these insertion sequences has been made possible by the development of techniques for the analysis of DNA structure. These techniques have shown that multiple copies of insertion sequences are normal constituents of the bacterial chromosome and also of extra-chromosomal genomes; their location on bacterial plasmids has suggested that they play a key role in chromosome evolution. Insertion sequences have been identified among the repititious DNA sequences which flank drug-resistance genes on bacterial plasmids, strengthening earlier evidence that the spread of multiple drug resistance among bacterial pathogens is a manifestation of genetic plasticity made possible by DNA insertions.

There are encouraging signs that studies of other properties of bacteria will benefit from the application of new technologies. Already, there is new information about mechanisms of attachment to mucosal surfaces, and the role of pili in such attachment. Studies, of chemotaxis, of enzymes, of membrane biogenesis, and of the structural dynamics and functions of outer membranes, are providing insights regarding bacterial virulence and the prospects for new bacterial vaccines. Work continued during the year on such vaccines. A polysaccharide vaccine has been developed and licensed for Groups A and C meningococci, but attempts to produce an immunogenic and protective Group B polysaccharide vaccine have failed. Groups B, C, and Y meningococci share an outer membrane protein antigen, type 2, and a type 2 protein vaccine is being developed for the prophylaxis of Group B meningococcal infections. Two proteins for the gonococcus -- the principal outer membrance protein and pili protein -- have been shown to be single proteins with only a small amount of lipopolysaccharide (endotoxin), and are ready for Phase I trials. Of several protein antigens from toxigenic E. coli, one -- described as "colonizing factor antigen" -- when used as a sub-cutaneous vaccine protected against illness and reduced intestinal colonization in initial volunteer challenge studies. Another antigen related to attachment -- pili -- will soon be tested in volunteers. Work is progressing on characterization of the antigens of Groups B streptococci as potential vaccine components.

Mycology

Systemic fungal infections have long been recognized as important causes of severe, sometimes life-threatening disease. Primary infections with two fungi -- Coccidioides immites and Histoplasma capsulatum -- are particularly common in the southwestern and central U.S., respectively. Other fungi are opportunistic pathogens, and infections with such organisms are being recognized with increasing frequency as the use of immunosuppressive therapies increases in the management of patients with malignant diseases or organ transplants. There are few effective drugs for the treatment of mycotic infections in patients with impaired immune defenses.

Consideration of these problems by an MIDP sponsored workshop led to the recommendation that several national mycology centers be established to assemble

a critical concentration of scientists knowledgeable in biochemistry, molecular biology and genetics, as well as in the clinical aspects of medical mycology, to provide a focus for contemporary research. Two institutions, the University of Washington, St. Louis, Missouri, and the University of California at Los Angeles, successfully competed for program project grants for the support of such mycology research units. It is believed that augmented research at these two strategically located units will revitalize the field of mycology and lead to more effective prevention and treatment of fungal infections.

Concurrently, multi-center clinical trials of antifungal agents in selected infections such as cryptococcal meningitis have gotten underway. In prospect is a controlled trial of a new drug, ketoconazole, a synthetic oral antifungal compound which inhibits the biosynthesis of ergosterol, the major sterol in most yeast and fungi. The drug apparently can be used without impairing cholesterol synthesis in man. It should be possible to design other such targeted approaches as more is learned about the molecular structure and dimorphism of the pathogenic fungi.

Parasitology

The new and expanded programs in international health and tropical medicine implemented this year through the support of collaborative research overseas (International Collaboration in Infectious Diseases Research - ICIDRs) and of multidisciplinary programs in the U.S. (Tropical Disease Research Units - TDRUs) will augment research on parasitic diseases, particularly those targeted by the World Health Organization: malaria, schistosomiasis, trypanosomiasis, leishmaniasis, and filariasis. This effort was facilitated by the many ties that University scientists have maintained with foreign investigators, and by the already existing studies of the Parasitic Diseases Panel of the U.S.-Japan Cooperative Medical Science Program. Four of six ICIDRs will be funded in FY 79; two ICIDRs and at least two TDRUs are projected for FY 80. Details are provided at the end of the Director's Report and in the report of the Molecular Microbiology and Parasitology Branch.

This increased effort comes at an opportune time. In preparation for a "Conference on Pharmaceuticals for Developing Countries" held in January, 1979, by the Institute of Medicine, National Academy of Sciences, a survey was made of current programs in U.S. government and academic laboratories for the development of preventive, prophylactic, diagnostic and therapeutic agents for the five parasitic diseases listed above plus leprosy and enteritis. In 1978, of the $36,013,000 spent by federal agencies for these purposes, $11,181,000 was invested by NIAID, $9,135,000 in behalf of extramural research. Considering the billions of world citizens infected with these and related diseases, an increasing number of whom are being offered refuge in the U.S., this is a sensible investment indeed.

Virology

One of the outstanding accomplishments of the year was the publication of the six volume report of the Virology Task Force, culminating two years of review by distinguished scientists for all parts of the U.S. who assessed the state of the art in virology and identified opportunities for fostering the under-

standing, prevention and control of viral diseases. Each of the five Task Force panels made a number of recommendations which were combined into a total of 25 in the final volume of the Report. These, in turn, were consolidated by the National Advisory Allergy and Infectious Diseases Council into ten research areas which, the Council recommended, should be emphasized and expanded by more than doubling the Institute's investment in research on virology in the next five years. That such an investment will yield significant dividends is buttressed by the Task Force's accounting of recent accomplishments and its projection of what lies ahead. As illustrated by paraphrased or excerpted sections of the report, the future is bright.

The history of virus research mirrors the progression from descriptive approaches to the era of molecular genetics. The development of improved biochemical and biophysical techniques for studying the structure and assembly of viruses has led to increased knowledge of the biosynthesis of viral components. This, coupled with the isolation of suitable mutants, has opened the way for a concentrated attack on the precise mechanisms involved in assembly processes and their regulation. Further efforts should lead to greater knowledge of important pathogens on the one hand, and of normal macromolecular and cellular organization and function on the other.

The fact that viruses, as bearers of unique genetic information encoded in their DNA or RNA, bring into cells conveniently small probes of essential life processes has made viral research the cornerstone of molecular biology. Viruses occur in all living creatures, and progress toward an understanding of those which cause disease in man has derived from concepts and techniques first applied to viruses of plants and bacteria. Viruses which infect bacteria are known as bacteriophages. Historically, the ideas of messenger RNA, of the triplet code of stop and start codons, even the very definition of a gene arose from their study. Future research is likely to yield results of importance to the medical community in many ways.

Temperate phages, along with plasmids, are members of a class of important genetic elements known collectively as "episomes." A recent finding in the field of episomal genetics has been that the genes which confer resistance to the antibiotics are carried on translocatable DNA elements capable of transposition from one bacterial genome to another. Thus, phage research is applicable directly to the mechanisms of pathogenesis in bacteria since many bacteria are dangerous only when they contain particular plasmids or phage genomes. Understanding the mechanisms of maintenance, immunity, incompatibility, gene expression, and the evolution of episomes will be of direct benefit just as it will be in the case of infectious drug resistance. One might even imagine devising competitor plasmids and phages which could effectively compete with undesirable plasmids or phages from the bacterial cell.

Ideas about the fundamental mechanisms of gene expression, recombination, replication, and evolution, as well as related technology derived from phage research, are often directly applicable to the study of animal viruses or other organisms. Genetic analysis of viruses will seek to determine the number of viral genes, the function of each gene, and the order or position of these genes on the nucleic acid chromosome of the virus. Studies involving the process of recombination will then position the genes relative to each

other, yielding a genetic map of the virus. Once this has been accomplished, several steps may be taken with viral genes of known function to elucidate the mechanisms of viral virulence or to produce candidate viruses or antigens for vaccine production. Mutations in viral genes can be sought which alter pathogenicity, e.g., by affecting temperature of growth, to produce live, attenuated vaccine strains. These viruses can then be used to induce immunity similar to that following natural disease, but without illness. Alternatively, the gene for an immunizing antigen, e.g., influenza virus hemagglutinin, may be transferred to an E. coli host via recombinant DNA technology, permitting replication of the gene and synthesis of the desired antigen as the bacterium grows in culture. Hepatitis and influenza genes are now being tested in such systems. Successful production and purification of specific antigens would have a great impact on vaccine manufacture and immunization practices for the prevention of acute viral infections.

Selected Acute Infections: Infections are the most common cause of illness; viruses are the most common cause of infections; respiratory viruses and enteric viruses are the most common causes of viral infections, ranking first and second, respectively, as causes of illness. Although considerable progress as been made in identifying the viruses responsible for these illnesses, they persist as major public health problems because of the inadequacy of knowledge essential to the development of effective preventive measures and therapy.

Acute respiratory infections are caused by a multiplicity of viruses, the most dramatic of which is influenza A, the cause of periodic world-wide-epidemics associated with high attack rates, significant morbidity and excess mortality. The most recent influenza A variant to appear -- in this instance, to reappear -- is the Russian strain (H_1N_1) which caused outbreaks in children and young adults in 1978 and 1979. In 1978, MIDP coordinated clinical trials of A/USSR influenza vaccine, combined with A/Texas and B/Hong Kong antigens, in approximately 2,100 subjects. Reaction and serologic data from these trials provided the basis for the formulation of vaccines and recommendations as to dosage schedules for the 1978-79 season. At a series of technical meetings during the past year, culminating with the Surgeon General's Meeting on February 12, and the Secretary's Conference on March 6, 1979, these data were updated for those developing immunization recommendations for 1979-80. These reviews were greatly facilitated by the availability of NIAID's own computer competence in the Epidemiology and Biometry Branch, and by the close working relationship of the three agencies responsible for surveillance (CDC), control of vaccine production (BoB/FDA), and research (NIAID). The Director's office emphasized this collaboration when it elected to display DHEW research on influenza according to Science Base, Application, Transfer, and Training, the SATT model. The Director, NIH, then requested that this model be used as a basis for drafting a DHEW trans-agency five year research plan for influenza as an example of health research planning.

The most important respiratory disease of children is that caused by respiratory syncytial (RS) virus. In infants and young children RS produces severe, sometimes fatal bronchiolitis. Older children and adults experience less serious illness during reinfection than do infants undergoing primary infection. Since serious RS virus disease occurs most often during the first few months of life, when infants possess passively transferred serum antibody, it is clear

that such neutralizing antibody in serum does not provide effective protection against the most serious effects of the virus. Nor, as noted, does it protect adults; reports during this year have documented the occurrence of moderately severe upper respiratory illnesses in adults exposed to children during the epidemics which occur every year in urban centers. Clearly, vaccines cannot be expected to prevent RS infection, so the current approach is to seek amelioration of the first infant illness by inducing local antibody with attenuated virus prior to infection with wild virus. Volunteer studies now in progress may permit this hypothesis to be tested in the coming year.

Acute viral gastroenteritis affects a broad segment of the population throughout the world. In the developed countries it is a major cause of morbidity in infants and young children, whereas in the developing countries it is a major cause of both morbidity and mortality in this same age group. Considerable progress has been made in elucidating the etiologic agents of viral gastroenteritis. Of note is the fact that these advances have been made without the use of classical cell culture systems which have been the keystone in the progress of modern virology but which are of no value in detecting these fastidious gastroenteritis agents in clinical specimens.

Two groups of etiologic agents of viral gastroenteritis -- the parvovirus-like group of which the 27 nm Norwalk particle is the prototype, and the 70 nm rotavirus group -- have been identified. Studies of rotaviruses have depended heavily on the use of the electron microscope (EM) and studies of the Norwalk group of viruses have relied exclusively on the electron microscope, utilizing almost always the technique of immune electron microscopy (IEM) -- a method which might be defined as the direct observation of antigen-antibody interaction.

It is evident now that a 70 nm rotavirus is a major etiologic agent of sporadic infantile gastroenteritis. This virus has been associated etiologically with up to 50 percent of the hospitalized cases of diarrheal illness in many developed countries in temperate climates. Viruses of the Norwalk group are being related to more and more local outbreaks. Currently, second generation tests which are being or have been developed offer great promise for further unravelling the natural history of these agents. Future studies will have to address (i) the efficient propagation of these agents in cell culture, (ii) a comprehensive definition of their overall importance over a sustained period in the etiology of gastroenteritis in various populations, (iii) elucidation of the immune mechanisms involved in host defense, (iv) the development of effective methods to prevent or treat illnesses due to these agents, and (v) the continuing search for other etiologic agents of actue gastroenteritis.

Similarly, the ability to recognize infections due to hepatitis A or B viruses has disclosed the need to research for other etiologic agents of hepatitis. The existence of "non-A, non-B" hepatitis viruses is inferred from the identification of hepatitis cases that lack serologic evidence of infection by hepatitis A or B viruses, cytomegalovirus or Epstein-Barr virus. Non-A, non-B hepatitis cases have been detected throughout the world. At present approximately 90 percent of post-transfusion hepatitis in the U.S. is type non-A, non-B. Recent studies of patients with repeated cases of apparently acute hepatitis suggested that at least two agents not related to type A or type B viruses exist, and recent transmission studies in chimpanzees have

provided electron microscopic evidence of two different viruses. Thus, with the prospects of an effective vaccine for hepatitis B, and the recent cultivation of hepatitis A virus, it is important to determine what proportion of hepatitis cases occurring annually in the U.S. is caused by as yet unidentified agents.

Influenza and other respiratory viruses are being studied at the Influenza Center and at several Vaccine Evaluation Centers; viral gastroenteritis is being studied at three Enteric Diseases Centers; viral hepatitis is being studied in several populations. There are, of course, many other viruses which infect man. A number of these infections can be related etiologically to specific agents, but, unfortunately, viruses have yet to be isolated from many presumed viral illnesses. The time is ripe for expanded epidemiologic studies of appropriate populations to define the natural history of these infections. In the view of the Virology Task Force this will best be done for the many widely prevalent viruses by continuing surveillance of family units. Although costly, relatively difficult, and requiring a long period of time to be productive, such family studies have two important advantages: (1) a given study can serve to describe the behavior of a large number of known viruses; and (2) it will yield a valuable "library" of specimens and illness information that can be exploited anew when new methods become available for studying infections with important new viruses or with currently known viruses which, for lack of readily applicable methods, have not yet been extensively studied. MIDP wishes to initiate one or more "Family Studies" as recommended by the Task Force.

Chronic Disease: It has long been suggested that viruses may cause chronic as well as acute diseases. It is now known that one or more of the viruses that cause actue hepatitis may persist and cause chronic liver disease. There are other viruses which induce no acute symptoms, but which as a result of "slow", progressive persistent infections, are responsible for such rare CNS syndromes as Kuru, Creutzfeld-Jakob, and Familial Alzheimer Diseases. Observations in the past year have contributed new knowledge regarding the role of viral infections in a major chronic disease, diabetes. These studies support the hypothesis that acute destruction of specialized tissue cells may lead to chronic loss of function of those cells, specifically the beta cells of the islets of Langerhans of the pancreas.

The common cold and influenza, along with measles, chicken-pox, and mumps, are examples of acute viral diseases from which, barring complications, patients recover. In rare instances, measles virus may cause a severe and fatal brain disease called subacute sclerosing pan encephalitis (SSPE). In other instances, not so rare, the chicken-pox virus may persist in ganglion cells of the nervous system and be reactivated to produce the painful lesions of herpes zoster. Mumps virus, which sometimes produces meningitis and pancreatitis, as well as parotitis, has been questioned as a cause of diabetes. While the association of mumps with diabetes has yet to be established, at least one virus now has been incriminated as a cause of juvenile onset diabetes in humans. This is a coxsackie B4 virus isolated by investigators at the National Institute of Dental Research from the pancreas of a previously healthy ten-year-old boy who died in diabetic coma ten days after the onset of symptoms of an acute febrile illness. Previous studies in animals had shown that Venezuelan equine

encephalomyelitis virus, encephalomyocarditis virus, reovirus type 1, and coxsackie B4 virus could induce diabetes in certain strains of mice. They do so by destroying the beta cells. Since it is these cells which produce insulin, their malfunction or destruction lead to a lack of insulin, and thus, to insulin dependent, or juvenile, diabetes mellitus. The coxsackie B4 virus isolated from the young boy produced diabetes in susceptible mice.

Many questions remain to be answered in FY 80 and beyond. Is human susceptibility genetically determined as is the case in mice? Antibodies to coxsackie B4 are present in about half the population; even more common are antibodies to mumps. Diabetes may occur in children who lack antibody to either virus. Perhaps initiation of juvenile-onset diabetes requires more than a simple virus infection in genetically predisposed individuals. Why do damaged beta cells not regenerate? Clearly, additional epidemiological studies are needed to examine these questions. Such prospective, longitudinal, multidisciplinary studies will be costly and demanding, but the answers they seek would form the basis of new approaches to prevention of a particularly devastating chronic disease.

International Health

International health programs are administered by Dr. Earl Beck, Special Assistant to the Director, MIDP, in conjunction with individual program officers and coordinators.

The International Centers for Medical Research (ICMR) that have been operative for nearly 20 years will be phased-out on or about May 31, 1980. The four institutions participating in the ICMR program are the Johns Hopkins University with an overseas base presently at the Gorgas Memorial Laboratories, Balboa Heights, Canal Zone; Tulane University with a research unit in Cali, Colombia; the University of Maryland with a base in Lahore, Pakistan; and the University of California with a unit in Kuala Lumpur, Malaysia.

ICMR Evaluation: The plan to evaluate the ICMR program with 1% set-aside funds was dropped because of prohibitive cost projections and timing. Since the ICMRs will be in their phase-out year, it was decided to make plans now to evaluate the ICIDR program in about three years.

International Collaboration in Infectious Diseases Research (ICIDR) is a new initiative in the Microbiology and Infectious Diseases Program. It is divided into Part A, International Program Project Grants, and Part B, International Exploratory/Developmental Research Grants. The research emphasis of the new ICIDR program is tropical infectious diseases and the immunology of these diseases. Special attention is given to the six diseases of the WHO Special Program for Research and Training in Tropical Diseases.

Part A, International Program Project Grants, embraces broadly based multidisciplinary research programs that have a well-defined central focus or objective, with major portions of the research being conducted overseas with an acceptable foreign affiliate. Fourteen applications were received and reviewed; nine were approved, and five disapproved. Of the nine approved, six were within the fundable range. The Institute plans to fund four of the

program project grants in late FY 79, and two in FY 80, depending on the availability of funds. The grantees will be collaborating with scientists in Brazil, Thailand, Sudan, Colombia, and Pakistan.

Part B, International Exploratory/Developmental Research Grants, encourages an individual investigator to develop a biomedical research program with an overseas affiliate, with most of the work being done in the host country. Thirty-nine proposals were received and reviewed; sixteen were approved, and twenty-three disapproved. Of the sixteen approved grants, five were within the fundable range. These grantees will be collaborating with scientists in Mexico, Brazil, India, and Nigeria. Based upon the Institute's Advisory Council's recommendation that the Part B portion of the ICIDR program be readvertized, consideration is now being given to this recommendation so as to provide applicants as much time as possible for the preparation of applications. Funding of these grants is projected for FY 81.

Diplomatic initiatives by successive U.S. administrations have involved NIH in a growing number of bilateral health agreements, with MIDP being assigned responsibility for coordinating participation of NIAID investigators in collaboration in infectious diseases research. The oldest and most productive of these bilateral programs is that with Japan, now completing its fifteenth year.

The U.S.-Japan Cooperative Medical Science Program provides the mechansim for scientists of the United States and Japan to collaborate on the following diseases or disease categories of importance to the health of the people of Asia: cholera, environmental mutagenesis and carcinogenesis, leprosy, malnutrition, parasitic diseases, tuberculosis, and viral diseases.

The Joint Subcommittee on Program Review and Planning met in Honolulu on February 8 and 9, 1979, and again on July 25, 1979, at NIH, just prior to the Fifteenth Joint Committee meeting. Major topics discussed and acted upon at the latter meeting included the following:

1. The formal review of the Joint U.S.-Japan Panels on (Methods for Evaluating) Environmental Mutagenesis and Carcinogenesis, initiated in 1978, was completed. The Committee accepted the report with revised guidelines as recommended by the Subcommittee. The future emphasis of this program will change from developmental methodology to population monitoring.

2. The formation of a Hepatitis Panel was formally approved along with appropriate guidelines and Panel members. The first joint meeting of the new panels will be held in Japan in 1980.

3. Revised guidelines for the Tuberculosis Panels were considered and approved. The guidelines were sharpened to reflect the heavy emphasis on research relating to the pathogenesis and immunological aspects of the disease.

4. Preliminary discussions were held to explore the possibility of introducing discipline oriented panels into the program, with the first such Panel to be one on Immunology. The matter is to be considered further at the next meeting of the Subcommittee on Program Review and Planning in February,

1980. The Subcommittee will make these recommendations to the next Joint Committee meeting in Tokyo in 1980.

5. A schedule was developed for publishing a Third Five Year Report, 1975-1980. A rough draft is to be ready for Subcommittee review at its February, 1980 meeting.

U.S.-U.S.S.R. Health Cooperation: The Institute's responsibility under this program consists of its participation in the U.S.-U.S.S.R. Collaborative Agreement on Influenza and Acute Respiratory Diseases. For the period October 8 through November 5, 1978, Dr. Fred Hayden, University of Virginia, participated in clinical trials of anti-influenzal drugs at the All Union Institute for Influenza, Leningrad. As part of a month-long visit to the U.S., Dr. Yuri Ivannikov of that Institute spent a week at NIH in March, 1979, discussing epidemiological studies.

As U.S. Coordinator of Problem Area IV - Chemotherapy and Chemoprophylaxis - Dr. George Galasso organized a meeting on this subject at the Influenza Center in Houston, Texas, on February 1-2, 1979. Five Soviet scientists participated, with three remaining for visits to U.S. laboratories. It is expected that several Soviet investigators will review the Russian experience with amantadine at the Consensus Conference on the use of this anti-influenzal drug scheduled for mid-October, 1979. Two days later, a joint meeting in Bethesda will address Problem Area II - Immunoprophylaxis - and its two topic areas. Dr. W. S. Jordan, Jr. is U.S. Coordinator for Topic 1, Development of killed and live influenza vaccines; Dr. Frank Ennis (BoB/FDA) is U.S. Coordinator for Topic 2, Standardization and control of influenza vaccines.

U.S.-China Cooperation in the Science and Technology of Medicine and Health: On June 22, 1979, representatives of the Ministry of Public Health of the People's Republic of China and of the Department of Health, Education, and Welfare of the U.S. signed a Protocol of agreement, and later participated in the first meeting of the U.S.-P.R.C. Joint Committee for Cooperation in Medicine and Public Health. One of the subjects designated for cooperative research is to be Infectious and Parasitic Diseases, with initial cooperation in four areas: 1) viral hepatitis; 2) schistosomiasis; 3) influenza; 4) malaria. Plans are now being made to implement this new bilateral agreement.

Cholera Research Laboratory: For nearly twenty years the NIH/NIAID has operated the Cholera Research Laboratory (CRL) in Dacca for the U.S. Agency for International Development (AID) as a field station for studying cholera and related diarrheas. The CRL was launched under the Southeast Asia Treaty Organization in 1960, and became the International Center for Diarrheal Disease Research/Bangladesh this year. This change in name reflects a change in administration negotiated by the CRL with its various sponsors. The final steps in the transformation of CRL to ICDDR/B occurred during the last week of June, 1979, when a new Board of Trustees met in Dacca and assumed responsibility for operating the Center. Dr. Carl Miller has served the U.S. administrative needs of the CRL for many years, and was particularly helpful during the recent period of transition. NIH will now relate to the ICDDR/B in its new status as an autonomous institution. It is anticipated that the laboratory will continue to make important contributions to the treatment and control of cholera and other diarrheal diseases.

BACTERIOLOGY AND VIROLOGY BRANCH

The Bacteriology and Virology Branch is responsible for the administration of a broad-ranging program of biomedical research; it supports program project grants, individual research grants, training grants, career development awards, individual postdoctoral fellowships, and contract programs of targeted research in bacteriology, virology, and mycology. Dr. Milton Puziss administers the Branch and the Bacteriology Program. Dr. William P. Allen administers the Virology Program, and also serves as Executive Secretary of the Virology Panel, U.S.-Japan Cooperative Medical Science Program. One of his recent activities, for which he received the NIH Director's Award, was bringing to a successful conclusion the work of the Virology Task Force. Dr. Darrel D. Gwinn administers the Mycology and Mycobacteriology Programs, and also serves as Executive Secretary of the Leprosy Panel and of the Tuberculosis Panel, U.S.-Japan Cooperative Medical Science Program.

Approximate Level of Support

Bacteriology and Mycology

Activity	Number	Amount
Research Grants	178	$12,821,120
Research Program Projects	7	2,167,800
Career Awards	11	399,169
Training Grants	19	1,563,342
Fellowships	10	142,200
Research Contracts	12	1,003,379
Total	237	$18,097,010

Virology

Research Grants	194	$16,016,949
Research Program Projects	2	464,087
Career Awards	21	699,908
Training Grants	13	1,177,476
Fellowships	17	211,150
Research Contracts	1	22,000
Total	248	$18,591,570

Branch

Total	485	$36,688,580

Program Summary

The program of this Branch focuses upon projects in both fundamental and applied biomedical research, with the ultimate objective being the translation of the knowledge gained into more practical methods for the diagnosis, prevention

and therapy of bacterial, fungal and viral diseases. Certain research areas have been considered as Programs of Institute Emphasis; research in these targeted endeavors is continuing and expanding where possible. These ongoing Institute Emphasis Programs are in Sexually Transmitted Diseases, Hospital Associated Infections, Streptococcal Diseases and Sequelae, and Mycology, plus several in Virology. Viral infections have a significant and continuing impact on the health of the public; control of these infections is a vital part of the Virology Program where studies in Persistent Infections and Chronic Viral Diseases, Clinical Virology, and Biology of Viruses have been highlighted for increased attention. Targeted research on rabies, dengue and other arthropod transmitted diseases under the auspices of the U.S.-Japan CMSP is also a part of the thrust toward expanding knowledge of viral diseases commonly found in the developing countries of the world.

Bacteriology

Sexually Transmitted Diseases (STD)

Sexually Transmitted Diseases continue as one of the most pressing problems in public health today, with little indication of any significant diminution in their prevalence. Gonorrhea is still the most important of these diseases, although many experts now consider that this has been superseded by the rapid increases in nongonococcal urethritis (NGU) and in herpes simplex genital infections (HSV-2). There is also increasing concern regarding other diseases, such as hepatitis, amebic dysentery, and shigellosis that are now being seen with increasing frequency, particularly among homosexual groups. The Institute's overall research program in support of the STD problem includes program projects (STD Centers), grants, contracts, and training. An important new thrust in FY 1979 was the initiation of a contract to develop candidate vaccine materials for the eventual control of gonorrhea.

Research Highlights

PO1 AI 12192-04 K. K. Holmes (University of Washington): Dr. Holmes, the director of this project, has reported that chlamydial infections in females have a strong association with neonatal death, in addition to fetal wastage and low birth weights. He also found that these infections may be a cause of peri-hepatitis (liver surface infection), as detected by a rise in antibody titers. Dr. Falkow of this group has found that Chlamydia trachomatis and C. psittaci have a group plasmid of apparently the same size, 4.5 daltons. This plasmid was also found in Lymphogranuloma venereum strains of Chlamydia; there is, therefore, a very close genetic relationship between these chlamydial organisms. The chlamydial plasmid DNA has been cloned into an E. coli plasmid; the role of this plasmid as a virulence factor in disease, however, is not yet clear. Dr. Eschenbach has undertaken a study of vaginitis as an STD problem. He has reported that Hemophilus vaginalis infections can be successfully treated with metronidazole (Flagyl). In a study of male partners of infected females, he isolated H. vaginalis from both the urethra and semen. He further noted that vaginitis may be a mixed infection with H. vaginalis together with an anaerobic flora. By DNA homology studies H. vaginalis was determined to be unrelated to Corynebacteria species or to any other similar organism -- it may be a new genus.

AI 13233-03 J. A. Yorke (University of Maryland): This investigator is studying the epidemiology of gonorrheal infections by developing a mathematical model of the existing and potential effectiveness of various control programs. His study indicates that screening programs for gonorrhea discover only one-tenth of all infected women, usually in the asymptomatic stage. He also emphasizes the importance of relatively small "core" groups who are repeatedly reinfected and are efficient disease transmitters. In a model based on use of a hypothetical gonorrheal vaccine administered to this "core" group, he reported that there would be a substantial reduction in gonorrhea prevalence, even with immunity of short duration. Dr. Yorke emphasizes that it is roughly three to five times as important to identify and treat the source of the primary infection as it is to treat a secondary infection. This concept of a "core" group of efficient disease transmitters can be valuable in understanding the transmission dynamics of other diseases.

PO1 AI 12618-04 J. M. Knox (Baylor College of Medicine): Dr. G. Dreesman, an investigator in this program project, is attempting to develop efficient techniques to purify native, immunologically active herpes virus type 2 (HSV-2) glycoproteins. Such a viral subunit would have significant potential as a candidate vaccine. Dr. Dreesman demonstrated that HSV-2 glycoproteins of 119,000 MW will induce virus neutralizing antibodies in immunized rabbits and guinea pigs. A new technique was developed for fractionation of viruses; distinct peaks were isolated, with MW ranging from 20,000-200,000. These were tested for HSV type-specific antibody by a microsolid phase radioimmunometric assay. The peak containing protein with 100,000-140,000 MW reacted preferentially with an HSV-2 antibody positive human serum, but not with an HSV-1 (oral herpes) serum. This material is now being evaluated for induction of protective antibody in guinea pigs. Other studies by this group show that 10% of females with prior HSV-2 episodes shed virus during their asymptomatic periods long after their overt disease phase, a finding of significant epidemiological importance relative to spread of the viral infection.

AI 14648-02 A. G. Plaut (New England Medical Center, Boston): Dr. Plaut previously reported the discovery of a protease enzyme in the gonococcus that destroys IgA immunoglobulin. Purification kinetics of this enzyme are under study. Antibody to the protease is inhibitory to the enzyme; patients with active gonococcal infection have a high titer of antibody to this protease. He has also found that this IgA protease occurs in Streptococcus pneumoniae and Hemophilus influenzae, in addition to the pathogenic Neisseria species.

Hospital Associated Infections (HAI)

Nosocomial, or Hospital Associated, Infections are an extremely troubling national health problem, resulting in significant mortality and excessive costs. As the immunocompromised patient population increases, the HAI problem tends to increase as well. The gram negative bacteria that are most commonly involved in these infections show a disturbing trend to antibiotic resistance, causing ever great difficulty in patient therapy.

Research Highlights

AI 10108-09 A. I. Braude (University of California, S.D.): Dr. Braude reported earlier on the efficacy of his J5 antiserum, obtained from human volunteers immunized with the "core" glycolipid from an E. coli strain, in lowering the death rate from gram negative bacteremia. Newer data with 50 of 90 additional patients (the study is still incomplete) indicate that the trend is very favorable, consistent with the life saving therapeutic effectiveness reported earlier. In these newer studies the prominence of bowel, lung, and urinary tract sites for gram negative infections was significant. The bacteremias were caused primarily by normal aerobic bowel inhabitants (E. coli and Klebsiella species) and antibiotic resistant opportunistic microorganisms (Pseudomonas and Serratia); Pseudomonas was the organism most frequently isolated from the blood. The prophylactic value of J5 antiserum was studied in agranulocytopenic rabbits to determine if it could prevent bacteremia. Results indicate that the intravenous injection of J5 antiserum protects rabbits against lethal Pseudomonas bacteremia; this protection lasts for at least six weeks. This study is being extended to determine the potential prophylactic efficacy of the J5 serum in burn patients.

AI 14533-01 R. Garibaldi (University of Utah): This project's goal is to establish cost-effective approaches to prevent post-operative nosocomial infections. A micropore membrane filter contact technique for quantitating bacterial contamination of wound surfaces was developed. This proved to be a sensitive and reproducible method for recovering bacteria; it may be an accurate predictor of wound colonization. In a retrospective comparison of hospitalization duration following post-operative infections, the data showed that wound infection rates varied with the type of surgery and likelihood of intra-operative bacterial contamination. The highest infection rate and prolonged hospitalization occurred following colon surgery (3.8%, with excess hospitalization of 23.8 days). This represents a significant addition to the costs of medical treatment. It was also shown that administration of antibiotics prophylactically prior to surgery was frequently misused. Such misuse probably predisposes to colonization and infection with antibiotic resistant bacteria. The routine use of an in-line micropore bacterial gas filter in anesthesia equipment had no effect on the incidence of post-operative pulmonary infection. Use of these devices is not cost effective and probably should be discontinued.

AI 13120-03 C. D. Cox (University of Iowa): Dr. Cox previously reported investigations on the iron-binding siderophore, pyochelin, of pathogenic Pseudomonas. He showed that all clinical isolates apparently possess this iron-binding pigment. Knowledge of the pyochelin structure is important for understanding iron metabolism and how to interfere with this effect during an infection. Most of the pyochelin structure has now been elucidated -- it is about 650 MW, composed of two identical subunits each containing a salicylic acid and sulphur. The compound is also very labile. The chelated iron is adsorbed rapidly to the bacterial surface; the pyochelin is exceptionally effective in stimulating iron uptake by the bacteria at extremely low concentrations. Pseudomonas bacteria passed through mice infected intraperitoneally are most responsive to host iron

for increased lethality; organisms harvested from lung tissue (intrathoracic infections), however, suppress phagocytosis and cause increased lethality. This mouse model describes differences in ecological niches in the host as well as mechanisms of Pseudomonas virulence.

AI 12936-03 J. W. Alexander (University of Cincinnati): Dr. Alexander has been studying immune responses in surgical infections. Previous data indicated that abnormal neutrophil function was clearly associated as a predisposing factor in post-surgical infections. This group is now studying the close association between nosocomial infection and nutritional status. Further data indicate that well-nourished burn patients supplemented with plasma did very little better than controls, but patients with a "high protein" regime showed a significant improvement in neutrophil function. In a mouse model system, administration of Corynebacterium parvum vaccine resulted in increased macrophage activation. The C. parvum vaccine also resulted in increased survival following challenge by Staphylococcus aureus and Listeria monocytogenes. Additional study in guinea pigs showed that skin grafts in cortisone-treated immunosuppressed animals had enhanced survival times when C. parvum was administered. These observations are of clinical significance in establishing a rationale for use of substances like C. parvum in treating infections in certain immunosuppressed conditions. The clinical aspects of these studies are now being investigated.

AI 11271-06 L. S. Young (University of California, L.A.): This investigator has focused on host interaction to Pseudomonas infection. He has reported that patients with advanced Cystic Fibrosis (CF) showed a high level of circulating immune complexes against Ps. aeuruginosa. Further these complexes had "enriched" antibody titers against lipopolysaccaride antigens of the organism. No significant antibody level against Pseudomonas exotoxin A in the CF patient sera was observed; preliminary data indicated, however, that the immune complexes contained endotoxin-like activity.

Conference

A workshop on Pseudomonas infections in Cystic Fibrosis (CF) was held on January 9, 1979, to explore the magnitude of this infectious disease problem. These infections are responsible, in large part, for the extreme disability and early mortality of the young CF patient. Recommendations for expanded research in selected areas of this disease problem were developed as a result of this meeting; publication of the summary proceedings will be in the Journal of Infectious Diseases.

Streptococcal Diseases and Sequelae (STP)

Group B streptococcal disease (GBS) persists as a significant cause of neonatal mortality or serious sequelae. Infants of mothers with low maternal antibody levels are at high risk of developing GBS infections. Research leading to a better understanding of the disease problem and to development of a candidate vaccine is of high priority.

Research Highlights

NO1 AI 72538 D. L. Kasper (Peter Bent Brigham Hospital, Boston): This contract is for study of the antigens of the GBS and development of a candidate vaccine to protect neonates at risk. The objective is to vaccinate pregnant women with low maternal antibody levels so that the neonate at birth will have a high protective maternal antibody. Research is focused on the capsular polysaccharide of the type III GBS. This purified polysaccharide elicited a significant antibody rise in immunized human volunteers, correlated with a rise in the opsonic index. The antigen has a sialic acid side chain attached to a carbohydrate "core"; the side chain is important in eliciting the protective antibodies. Studies of GBS types Ia and II polysaccharide antigens have also been initiated. It is expected that eventually a tri-valent GBS vaccine will be developed, incorporating these three antigenic polysaccharides. Investigation has also begun on plasmaphoresis procedures to prepare high titer human immune globulins (IgG) from human volunteers. If successful this material will be used for passive immunization to protect high risk infants against the late-onset type of GBS disease.

AI 13150-03 H. R. Hill (University of Utah): Host-defense mechanisms against GBS infections are not fully defined. Dr. Hill has developed a promising rat model for study of GBS pneumonia. Newborn rats (under 16 hours old), inoculated with GBS type III intranasally (LD_{50} of $5x10^6$ organisms) developed fatal pneumonia and sepsis. Adult animals with similar challenge showed no bacteremia or mortality. Human serum containing GBS opsonic activity, when given intraperitoneally, protected challenged neonatal animals. The routes of infection, rapid death, neutrophil changes, and neonatal susceptibility are important parallels to human infection. When whole blood containing heat-stable antibody to the infecting GBS was given to human neonates, a rise in neonatal opsonic activity resulted. Nine of nine infants receiving this transfusion survived their septic episodes, whereas three of six infants receiving blood lacking antibody to their infecting strain died.

AI 14361-02 J. B. Zabriskie (Rockefeller University): This project is a study of the streptococcal and host factors that are of pathogenic importance in the sequelae of streptococcal infection. A unique population in Trinidad is under study, where both rheumatic fever (RF) and acute glomerulonephritis (AGN) are common sequelae of the streptococcal infections. Results show that patients with RF respond primarily to antigens present in the protoplast membrane of streptococcal strains associated with RF and do not react to antigens found in strains associated with nephritis. Those strains associated with AGN secrete a protein antigen found in the streptococcal cell membrane; this antigen is also detected in patients' glomeruli. Each patient group thus reacts specifically to those antigens present in streptococcal strains associated with a particular disease. This patient reactivity may be genetically inherited.

Other Disease Problems

Staphylococcus aureus is an organism that can cause a number of distinct disease syndromes within the same host; the organism also produces an impressive array of biologically active toxins.

AI 14998-02 M. E. Melish (Kapiolani-Children's Medical Center, Honolulu): Dr. Melish is investigating the clinical-pathological correlates of infection with staphylococcal exfoliatin, or epidermolytic toxin (ET), the cause of staphylococcal "scalded skin syndrome" (SSS) in infants. A newly-developed antibody radioimmunoassay was 1,000 times more sensitive than earlier methods for detection of ET in blood tissues; this method detected ET in acute sera from all SSS patients tested. No free ET was detected, however, in patient sera after antibiotic therapy was begun. In an adult mouse model, epidermolysis by ET could be induced regularly; this developed 10-16 hours after peak serum levels of ET were established. Patients with generalized SSS showed no evidence of pre-existing ET neutralizing antibody. In contrast to generalized toxemic SSS, localized bullous impetigo can develop and progress despite high levels of circulating antitoxin. Screening of normal adults revealed that over 85% of those over age 20 have ET antitoxin, whereas only 24% of children under age two years do so. Thus, lack of this age-related antitoxin appears to be associated with the prevalence of SSS in children.

AI 07826-10 F. A. Kapral (Ohio State University): In this study on SSS, Dr. Kapral showed that the syndrome is the result of the intercellular separation among cells of the granular layer; it does not involve actual cell destruction. In a neonatal mouse model, he showed that a positive Nikolsky sign (loss of skin elicited by gentle stroking) cannot serve as the sole criterion for exfoliatin action. Recent data show that certain S. aureus strains produce a substance causing a positive Nikolsky sign distinct from exfoliatin. This substance caused destructive lesions beneath the granular layer; it also differed in antigenic specificity from ET. S. aureus strains isolated from patients produced this new exfoliatin-like material. Additionally, an extractable lipid-like material found within abscesses exhibited marked bactericidal activity against certain S. aureus strains.

AI 03772-19 P. A. Murphy (Johns Hopkins University): The pathogenesis of fever is only vaguely understood. Dr. Murphy has shown, in a goat model, that purified macrophages make at least four distinct pyrogens; these are in response to stimulation by labeled endotoxin, by labeled staphylococcal organisms or by influenza viruses. The purified pyrogens all gave double-labeled peaks regardless of the stimulating source. One of these macrophage pyrogens is identical to that from neutrophils; the other three are clearly different.

AI 09366-09 T. A. Stamey (Stanford University): Urinary tract infections in women represent a costly public health problem. Dr. Stamey has found that cervicovaginal antibody in female volunteers was directed against a variety of E. coli strains but not against the volunteers' indigenous flora. Antibody was uniformly found in salivary fluid, eliminating the possibility

of an immunodeficient reaction at mucous surfaces; bacterial pili, however, played a significant role in attachment to vaginal epithelial cells. The defect allowing E. coli attachment and disease initiation is most probably a local immunologic one in vaginal secretions.

Leprosy

Leprosy is found in almost every country in the world; it is a significant health problem in the developing countries of the world. The majority of the estimated 12 to 15 million cases of leprosy (10 million cases are recorded at WHO) are found in Asia, Africa and South America; as many as 2,000 cases are in the United States. Approximately 150 new cases of leprosy are reported in the United States yearly, with about 25% of these occurring in native-born Americans. More significant than the actual number of leprosy cases is the evidence that not more than 20% of the estimated cases in the developing countries are under regular treatment. Also, children make up roughly one third of the total number of those people infected with leprosy. Because of its worldwide importance, leprosy research continues to be supported by NIAID under the auspices of the U.S.-Japan Cooperative Medical Science Program.

Research Highlights

AI 10094-09 W. E. Bullock (University of Kentucky): Dr. Bullock has been concerned with further studies on the nature and function of suppressor cell populations generated within spleens of mice experimentally infected with Mycobacterium lepraemurium. Most recently, he has investigated the nature of splenic suppressor cells from infected mice that suppress cell mediated lymphocytotoxic activity by normal spleen cells. Through these studies it has been demonstrated that two such suppressor cell populations are generated within the spleens of M. lepraemurium infected mice. One suppressor population is composed of T lymphocytes and the other of macrophages or, possibly, B cells. The activity of the two populations acting together in whole spleen cell preparations usually is greater than the activity of either cell population alone.

AI 08417-10 A. H. Fieldsteel (Stanford Research Institute): Dr. Fieldsteel has continued his studies utilizing the neonatally thymectomized Lewis rat (NTLR) as a model for detecting persistent M. leprae. These experiments have demonstrated the superiority of the NTLR over mice for the detection of small numbers of viable M. leprae. Normal mice are routinely utilized to monitor the efficacy of leprosy chemotherapy. However, the maximum number of M. leprae that can be inoculated into mouse foot pads to demonstrate multiplication is 10^4, and the ceiling of multiplication is approximately 10^6 On the other hand, the NTLR can be inoculated with up to 10^7 M. leprae and the ceiling of multiplication is 10^8 to 10^9 organisms. These experiments demonmonstrate that the NTLR can be used as a model of M. leprae persistence.

AI 07801-12 J. L. Krahenbuhl (U.S. Public Health Service Hospital, San Francisco): Triatoma barberi (a blood sucking bedbug) was evaluated to determine the period of viability of M. leprae following ingestion of approximately 6×10^7 organisms suspended in rabbit blood. Inocula of 5000 M. leprae

organisms recovered from the Triatoma between one hour and four days after ingestion were viable and found to multiply in mouse foot pads to a maximum of 1 x 10^6 organisms; M. leprae recovered seven days after ingestion by Triatoma multiplied to only 3 x 10^4 organisms, and after 14 days no multiplication was observed.

Tuberculosis

In 1978, 28,521 cases of tuberculosis were reported to CDC. This represents a decrease, since 1977, of 5.4% in the number of cases reported. Since antibacterial drug therapy for tuberculosis was initiated, a shift in tuberculosis victims from young adults to older people has taken place. The atypical mycobacteria are related, but not identical, to Mycobacterium tuberculosis; although diseases caused by atypical mycobacteria have symptoms similar to tuberculosis, the infections are not transmitted from man to man. Also, the atypical mycobacteria are frequently resistant to the antibacterial therapy employed in treating ordinary tuberculosis. The number of cases of tuberculosis and atypical tuberculosis in the U.S. is small in comparison to the millions of cases found in the developing nations of the world. Tuberculosis in these countries remains one of the major causes of morbidity and mortality in all age groups. Tuberculosis research continues to be supported under the auspices of the U.S.-Japan Cooperative Medical Science Program.

Research Highlights

AI 13813-03 H. Gruft (New York State Department of Health): Dr. Gruft is surveying the estuaries in the eastern U.S. to establish a basis for understanding the epidemiology of atypical mycobacteria in relation to their geographical distribution. He has found a higher concentration of Mycobacterium avium-intracellulare-scrofulaceum (MAIS) strains along the Gulf Coast and in the waters of the Carolinas and Georgia. The highest concentration of MAIS strains are found in waters of 1-2% salinity, i.e., estuaries and mouths of rivers. MAIS strains are found in high concentration in waters from regions where most of the cases of positive skin tests have been found. They can also be found in the air in droplets small enough to enter the alveoli.

AI 11807-04 B. M. Sultzer (SUNY Downstate Medical Center): Since Dr. Sultzer's original discovery that PPD-tuberculin is a polyclonal activator of B-lymphocytes from uninfected or unimmunized mice, other laboratories have found PPD to be a useful probe for studying a variety of basic immunological problems. Further studies on the nonspecific activation of lymphocytes have shown that PPD can act as a polyclonal activator (PCA) of human peripheral blood lymphocytes; that PPD can act as an adjuvant and as a PCA in vitro and in vivo; and that PPD stimulates B-cell hyperplasia when directly injected into mice. Continuing studies have shown that this intrinsic biological property of PPD to activate B-cells is due to tuberculoprotein and not to extraneous materials. Fractionation studies indicated that the antigenic and mitogenic activities reside together in several molecular species of tuberculin proteins. Antigens without mitogenic activity can be separated from PPD; however, mitogens without antigenic activity have not been separated from PPD as yet.

NOl AI 02079 J. K. McClatchy (National Jewish Hospital): Laboratories throughout the world have sent Dr. McClatchy in the last year alone almost 1,400 isolates of atypical mycobacteria for classification and identification. In addition to his work with these cultures under the contract, he has supplied a number of investigators in the U.S. and abroad with reagents to perform their own serological identification. He has also investigated the chemical structure of the antigens which are used to classify the various atypical mycobacteria into different species and subspecies. The development of this methodology has been very helpful; Dr. McClatchy can now biochemically identify certain members of the atypical mycobacteria which could not be taxonomically classified using serologic methodology. Current work is being directed toward the identification of atypical mycobacteria found in pathologic specimens from diseased individuals.

Mycology

Primary fungal pathogens that are directly involved in pulmonary diseases, such as Coccidioides immitis and Histoplasma capsulatum, are well established as significant causes of morbidity and mortality, especially in well defined geographic areas of the U.S. Other fungal pathogens, such as Candida and Cryptococcus, are important as opportunistic microorganisms, particularly in patients with impaired host defense mechanisms. Treatment of these fungal infections requires prolonged administration of relatively toxic drugs and the therapy may not always be effective. The Institute is presently funding research in which these pathogenic fungi and others, including Blastomyces and Aspergillus species, are being investigated. The Institute has established a special program in Mycology to intensify research efforts in this area.

Research Highlights

TOl AI 00459-06 G. Medoff (Washington University): Dr. Medoff's group has been studying the control of dimorphic transition in fungi. H. capsulatum, an important human pathogen, is one of these dimorphic fungi. Its saprophytic form is mycelial and is found in nature, whereas the unicellular yeast is the parasitic phase found in infected tissues. One phase can be induced to transform to the other in culture when the temperature of incubation is adjusted to an appropriate level. An understanding of the biology and genetic regulation of the process, it is believed, will provide insights into the pathogenesis of disease caused by dimorphic pathogens and may help in the control of these infections. The major differences found between the two forms of this fungus were:

(a) Discovery and purification of a cystine reductase present only in the yeast phase of H. capsulatum. This enzyme and a cystine permease appear when the temperature is switched from 25C to 37C. Both enzymes may be involved in providing the sulfhydryl groups (in the form of cysteine) that are necessary to initiate and maintain the yeast phase.

(b) The level of cyclic AMP is approximately five times higher in the mycelial phase than in the yeast phase of H. capsulatum. Furthermore, addition of either cyclic AMP or inhibitors of cyclic AMP

phosphodiesterase induces transformation of yeast to the mycelial phase even at 37C, the nonpermissive temperature for mycelial growth in nature.

(c) The yeast and mycelial phase each have three RNA polymerases, similar to those in other eukaryotic cells. In H. capsulatum, the enzymes in one phase are clearly different, functionally and structurally, from the enzymes in the other. This represents the first case in which a clear difference in RNA polymerases has been shown to exist between morphologic phases of the same eukaryotic organism.

Dr. Medoff is now testing, with several mutants of H. capsulatum, a working hypothesis related to the sequence of events and control mechanisms in this transition.

K04 AI 00325-05 R. D. Diamond (Boston University School of Medicine): Previous studies demonstrated that neutrophils could attach to, damage, and probably kill Candida albicans hyphae by a nonphagocytic mechanism. In contrast to most phagocytic systems, attachment and spreading of neutrophils over live hyphal surfaces with activation of microbial mechanisms occurred in the absence of serum. Addition of IgG from human serum augmented the process. In the absence of serum, neutrophils did not attach to killed hyphae. However, supernatants from actively growing hyphae or hyphae killed with ultraviolet light inhibited attachment of neutrophils to live hyphae. Evidence from Dr. Diamond's current work suggests that the soluble inhibitor acts directly on neutrophils; however, the neutrophils are not significantly damaged by exposure to the inhibitory substance.

AI 13770-03 M. H. Weiner (University of Texas): The objective of Dr. Weiner's project is to develop antigen immunoassays to diagnose systemic fungal infection. Dr. Weiner has developed radio labeled antigen-binding inhibition assays (RIAs) to detect Aspergillus and Candida antigenemia. He has now analyzed the effectiveness of the RIAs with sera obtained from hospital patients with systemic bacterial and fungal infections and from normal donors. Using the RIAs he was able to detect accurately Aspergillus and Candida antigenemia in the sera from patients suffering from these fungal infections. This is the first demonstration of Aspergillus antigenemia detected in human sera obtained antemortem.

AI 05022-15 G. S. Bulmer (University of Oklahoma): Cryptococcosis, a frequently fatal meningeal fungus disease of man, is caused by the heavily encapsulated yeast Cryptococcus neoformans. Dr. Bulmer's goal is to determine why and how certain individuals contract this disease. His studies, with the intragastric inoculation (mice) of C. neoformans, indicate that medical personnel should be alerted to the possibility that cryptococcosis may begin in the gastrointestinal tract; i.e., cryptococcosis may not always originate in the lungs. Additionally, these studies indicate that the mouse, and possibly other rodents, may act as vehicles for dissemination of the pathogenic yeast in nature. Heretofore, pigeons have been considered to be the sole natural source for this organism. In another study Dr. Bulmer has shown that an aqueous extract of garlic is a potent killing agent of C.

neoformans. This material should be investigated further as a possible therapeutic agent against cryptococcosis and other mycoses.

Virology

Program Summary

Viruses continue to be a major cause of human illness in nearly all parts of the world, but modalities for control and treatment of these pathogens remain relatively limited. The large number and diversity of viruses that impact on human health add to the complexity of the problem of their control. Significant advances have been made in unraveling the components of viral structure, replication and interactions with the afflicted host, but much remains to be learned before a conquest of viral diseases can be attained. The Virology program within the BV Branch encourages and supports multidisciplinary approaches to research on viruses and viral diseases through its subprograms on Clinical Virology, Persistent Infections and Chronic Viral Diseases, and Biology of Viruses.

Clinical Virology

Clinical Virology is a program of Institute emphasis focused on pathogenesis and immunopathogenetic mechanisms of viral diseases of man. Clinically relevant research on pathogenetic viruses is encouraged to bridge a widening gulf between molecular-oriented virologists and clinical investigators specializing in infectious diseases. Research projects on herpesviruses are especially prominent, a reflection of the high incidence and severity of human diseases caused by herpes simplex viruses, varicella-zoster virus, cytomegalovirus and Epstein-Barr virus.

Research Highlights

AI 14341-02 L. Aurelian (The Johns Hopkins University): It is well established that human infections with herpes simplex virus (HSV) elicit both specific humoral and cellular responses. Dr. Aurelian has obtained preliminary data supporting the hypothesis that patients with a history of recurrent lesions suffer from an impaired generation of effector lymphoid functions (differentiated lymphoid cells that react specifically in response to exposure to viral antigens). The hypothesis further suggests that various HSV antigens may induce different responses, that such responses are affected by clinical status (vis., quiescence versus recrudescence), and that various in vitro assays measure the expression of different populations of lymphoid cells. In support of the hypothesis, recent results indicate that maximal effector levels are reached shortly after onset of recrudescence, i.e., during convalescence. They begin to wane thereafter, with borderline to negative levels being maintained during quiescence. New recrudescences occur on this background of low (or negative) effector functions. The phenomenon is virus specific.

AI 14564-02 R. R. McKendall (University of California, San Francisco): In the presence of specific antibodies, herpes simplex virus (HSV) becomes localized to specific sites in the body and is eventually eliminated or reduced to a

state of latency. Dr. McKendall has been investigating the characteristics of specific antibody molecules responsible for this defense against infection in a murine model. He has found that the neutralizing fragment, Fab, of the antibody failed to avert disease, which indicates that neutralization alone is not the major effect of antibody defense against herpetic infections. The results further suggest that effective control of viral spread within tissues is dependent upon the Fc fragment of the antibody molecule. The Fc fragment is not antigen specific. Moreover, if destruction of the blood-brain barrier is a late event in the course of herpes encephalitis or herpes myelitis, then serum neutralizing antibody would not be expected to be much of a host advantage. This suggests a new therapeutic approach through methods which would enhance both antibody and cellular penetration into the central nervous system early in the course of disease.

AI 14373-02 J. A. Zaia (Sidney Farber Cancer Institute): Infections with varicella-zoster (VZV) exert deleterious effects on the clinical course of immunosuppressed patients, particularly those with lymphoproliferative disorders, causing significant morbidity and occasional death as well as interrupting scheduled chemotherapy. Dr. Zaia's research is aimed at understanding the pathogenesis of VZV infection in these patients, including an understanding of the elements of immunity which may be defective during cancer therapy. He has developed the methodology for efficient identification of human plasmas containing VZV-immune globulin by means of screening large amounts of recovered plasma for specific antibody to VZV. In recent studies he has demonstrated that this immunoglobulin (VZIG) prevents chickenpox in immunosuppressed children. The VZIG was as effective as standard zoster immune globulin (ZIG) in preventing severe complications due to chickenpox. The advantage of VZIG is that it can be produced easier and made more available than ZIG. He has also developed a new laboratory method for the rapid detection of VZV viremia during zoster infection. The method utilized the Fab component of VZIG for the detection of VZV membrane antigen in peripheral blood mononuclear cells. These results also indicate that specific antiviral antibodies constitute the major defense against VZV infections in man.

AI 01475-22 E. H. Lennette (State of California Department of Health): Antibody against varicella-zoster virus (VZV) is routinely detected by complement-fixation (CF), or immune adherence hemagglutination (IAHA) or neutralization. Although neutralization is the most meaningful test in terms of protection against chickenpox or zoster, it is extremely cumbersome and expensive. Investigators in Dr. Lennette's laboratory compared the newer techniques of enzyme immunoassay (EIA) with neutralizing, IAHA, and CF and found that EIA offers a rapid, sensitive, and specific method of antibody assay that is applicable in a clinical setting. The EIA test had a 94% correlation with neutralization and could reasonably replace the neutralization test and reduce costs for serodiagnosis of varicella or zoster.

AI 11788-09 D. M. Horstmann (Yale University): In general, the presence of antibody to rubella virus detected by hemagglutination-inhibition (HI) tests indicates that the person has protective immunity. However, recent evidence obtained by Dr. Horstmann demonstrated that, in a few individuals naturally infected or vaccinated, the presence of HI antibodies in the absence of detectable neutralizing antibodies (NT) did not prevent reinfection.

Therefore, the NT test is the one of choice for assay of protective levels of antibody in vaccinees. The study showed that NT responses to the new RA 27/3 vaccine were higher titered and persisted longer at high levels than with the currently used HPV77DE5 and Cendehill vaccines. NT titers at 3-5 years post-infection were lower in vaccinees than in natural immunes. At nine years post-vaccination, 11% of 290 children who received the HPV77DE5 vaccine were found to be antibody negative by HI, and all of these had NT titers below protective levels. The results point to the superiority of the RA 27/3 vaccine, which will soon become available in the United States.

Workshop on Cytomegalovirus Infections during Organ Transplantation

As the import of cytomegalovirus (CMV) infection has become evident, improved techniques to study the molecular biology of this agent have been developed, and a variety of possible therapeutic interventions has been proposed that might be useful in preventing and/or treating this infection. In an effort to coordinate research efforts aimed at the control of CMV infection during transplantation and to evaluate those therapeutic and preventive modalities currently available, a workshop was convened in June 1978 by NIAID comprised of a small group of experts in the field of CMV who were intimately involved and concerned with clinical transplantation. The goal of the workshop was to summarize presently available information, and to prepare an agenda which would lead to the systematic application and evaluation of each of the therapeutic modalities discussed. A summary of the proceedings has been published in the Journal of Infectious Diseases.

Persistent Infections and Chronic Viral Diseases

The mechanisms are little understood by which some viruses can infect a host and then remain within the host in a quiescent state for years or slowly proliferate, eventually to cause chronic disease. It is speculated that many chronic diseases of undetermined etiology may be caused by viruses with unique capabilities of escaping the host's defenses. The Virology program has encouraged investigations on persistent and slow virus research in efforts to identify and characterize the putative agents. Current emphasis in this program has shifted from the search for additional agents to more complete characterization of known persistent and slow viruses and their pathogenetic mechanisms.

Research Highlights

AI 06246-15 J. G. Stevens (University of California, L.A.): Dr. Stevens has been studying the pathogenesis of herpes simplex virus (HSV) in model laboratory and animal systems. Until recently, he was not able to establish a latent infection in mice similar to that which occurs naturally in man. Dr. Stevens has now characterized 15 mutants of HSV with respect to their ability to become latent in mice. Seven of the mutants did become latent whereas eight did not. Latency did not depend on the ability of the mutant to replicate DNA within infected cells, but did appear to be dependent upon viral protein syntheses beyond the immediate-early polypeptide. The discovery of these latent mutants promises to make studies of latency and reactivation at the molecular level in model HSV systems more feasible than previously

possible. It is also significant that latency can be determined by the viral genome. It should now be possible to identify the locus and defect in the viral genome which regulates latency and, ultimately, to explain the mechanism.

AI 12438-05 R. M. Welsh (Scripps Clinic and Research Foundation): One of the prime models for study of persistent infections is lymphocyte choriomeningitis virus (LCMV) in mice and cell culture. Dr. Welsh has been investigating the relationships between the immune response and defective interfering (DI) particles in the maintenance of chronic virus disease. Particular emphasis has been on the role of natural killer (NK) cells in response to infections with LCMV. It has been shown that cells infected with DI synthesize viral proteins at a very slow rate. The decrease in expression of viral antigens could help to explain how these persistently infected cells escape immunologic attack mechanisms of the host. Dr. Welsh has also shown that acute infection with LCMV induces an augmented NK cell activity that is correlated with the synthesis of interferon type I (antiviral), and that NK cells were activated in vivo by interferon injections. Thus, these NK cells may be of great importance in controlling virus infections and contributing to immunopathology in acute and chronic infections. DI particles may be of significance in chronic infections by regulating viral antigenic expression, thereby limiting the severity of immunopathologic disease.

Biology of Viruses

Acquisition of knowledge concerning virus structure, function and interaction with the host has required technologies developed by several scientific disciplines including microbiology, biochemistry, biophysics, genetics, physical chemistry and immunology. The field of biology of viruses is very fluid, and is often found moving in the direction of a new technology. Current trends reveal a substantial movement toward such technologies as recombinant DNA, recombinant RNA, oligonucleotide fingerprinting, and nucleotide and peptide sequencing to approach questions yet to be answered about the composition of viruses and how they infect cells, replicate and cause disease.

Research Highlights

AI 12717-04 E-S. Huang (University of North Carolina): Dr. Huang, in collaboration with Dr. C. Alford, University of Alabama, has been analyzing by restriction enzyme cleavage strains of cytomegalovirus (CMV) isolated from patients presenting various clinical manifestations such as recurrent infection, congenital defect and mononucleosis. The objectives of the study are to understand the mode of viral transmission, to classify viral strains, and to identify the origin of CMV isolates. In comparing viruses isolated from five sets of mothers and their offspring the restriction enzyme-fragment patterns were found identical even though some of the offspring viruses were isolated several years after virus was isolated from the mothers. In one recurrent case the fragment patterns of original and recurrent isolates were identical, indicating that the two isolates were derived from the same parental strain. In another recurrent case, the recurrent isolate was distinctly different from the original, suggesting that the recurrent infection was caused by a new virus strain. In other collaborative studies, Dr. Huang has demonstrated that CMV virus isolated from an immunosuppressed renal transplant patient is different from

the CMV virus used to immunize that patient before the transplantation. This is an important observation because it indicates that the CMV used for vaccination was not reactivated after transplantation and during immunosuppression. Rather it suggests that the post-transplantation infection was probably derived from the donor kidney.

AI 14049-02 L. C. Norkin (University of Massachusetts): SV 40 virus is an agent found to persistently infect kidney cells of rhesus monkeys. First discovered as a contaminant of early polio vaccines, it has become an excellent model for study by molecular virologists because of its simplicity, its ease of propagation and its oncogenic properties. Dr. Norkin has been investigating variants of this virus that arise during persistent infections with respect to properties that might affect the course of infection. He has shown that stable persistent infections of rhesus monkey kidney cells can be established with little, if any, visible damage to cells. Only about half of the kidney cells are initially susceptible to infection, but after three to 11 weeks all cells contain and express virus genetic information. Only a small percentage of persistently infected cells produce infectious virus and these eventually die. The virus-producing cells are eventually killed and the productive infection is perpetuated by the emergence of new virus-producing cells from the population of nonproducing cells. Defective viral particles eventually emerge which interfere with the replication of infectious virus. Dr. Norkin has demonstrated that this interference is not associated with interferon.

AI 12458-05 F. B. Bang (The Johns Hopkins University): Dr. Bang's laboratory has been investigating the effect of diet on susceptibility to viral infections. They have focused their studies on vitamin A deficiences in chickens infected with Newcastle disease virus (NDV), and on low protein diet in genetically susceptible and resistant strains of mice infected with mouse hepatitis virus (MHV). Results have demonstrated a synergistic effect of NDV and vitamin A deficiency in producing massive destruction of the bursa and thymus (two major organs of the immune system) in the chicken. Similar effects on the immune system were found with low protein diets and MHV in susceptible mice. The mouse system proved more amenable than the chicken for study at the cellular level in vitro. In this context, recent work has shown that in terms of susceptibility to MHV, the behavior of the macrophages in genetically resistant and susceptible mice mirrors both the genetic and phenotypic susceptibility of the intact mouse. These systems in the chicken and mouse promise to provide models for a better understanding of the effects of diet deficiencies on susceptibility to infectious agents.

AI 09706-09 H. Koprowski (The Wistar Institute): The new technology of using hybridoma cells to produce monoclonal antibodies is opening new approaches to the study of viral proteins. In this laboratory the technique was applied to the study of proteins of rabies virus. Cultures of hybridoma cells prepared by fusing mouse myeloma cells with spleen cells from rabies-immunized mice resulted in numerous clones producing highly specific antibodies. The antibodies produced by over 100 clones were analyzed and divided into the categories of specificity corresponding to the known rabies virus antigens. Because of the highly specific properties of these monoclonal antibodies, the investigators were able to differentiate strains of rabies virus antigenically.

Previous to these observations, it was generally believed that all strains of rabies were antigenically very closely related. The monoclonal antibodies have also substantiated that antibodies against the glycoprotein of rabies virus are responsible for neutralization of viral infectivity and for immune lysis of infected cells.

Virology Research under the Auspices of the U.S.-Japan Cooperative Medical Science Program

Goals for this program include the encouragement and support of research that will advance our knowledge of and lead to eventual control of rabies, dengue and other arthropod diseases and viral gastroenteritis. The program is monitored by the Panel on Viral Diseases, an advisory group to the U.S.-Japan CMSP.

Research Highlights

Rabies

AI 09706-09 H. Koprowski (The Wistar Institute): The new WI-38 human diploid cell rabies vaccine (HDCV) developed at the Wistar Institute is now produced in both France and West Germany and is licensed for pre- and post-exposure human prophylaxis in several European and Asian countries. The licensing by Wyeth Laboratories, Philadelphia, of HDCV produced in the U.S. is still in progress. The necessary clinical investigations have been completed; production will be initiated immediately in newly constructed facilities. The license is expected to be granted after completion of the first four lots of vaccine. In the meantime, the HDCV is widely used as an Investigational New Drug (IND) with the authority for distribution in the Center for Disease Control, Atlanta. Over the last year, several hundred people in the U.S. were successfully treated with HDCV.

Conferences

Workshop on St. Louis Encephalitis

A workshop convened in June 1979, under the auspices of the U.S.-Japan Cooperative Medical Science Program, was held at the Rocky Mountain Laboratory in Hamilton, Montana. The state of the science of St. Louis encephalitis (SLE) virus was reviewed and potential areas for future research were discussed. It was the consensus of the participants that substantial new information has been obtained on the biology and biochemistry of SLE virus, and exciting new research on flaviviruses is in progress. Research leading toward an SLE vaccine was encouraged, but reservations were expressed as to the advisability of developing a vaccine now before there is sufficient information on the protective response to SLE in man. Potential control of SLE through a weak link in its mosquito vector transmission was discussed, i.e., the emerging female mosquito after overwintering. More complete information on the ecology of overwintering vectors is needed to locate sites where control measures would be most effective. A summary of the workshop will be published in the Journal of Infectious Diseases.

CLINICAL STUDIES BRANCH

The Clinical Studies Branch (CSB) serves as the Institute's focus for the development and processing of Investigational New Drug Applications, and maintains liaison with the NIH Human Research Review Panel and the NIAID-Clinical Research Subpanel. It provides and monitors a closed clinical facility for Institute volunteer studies, and conducts, promotes and supports the development and evaluation of procedures for the diagnosis, prevention and treatment of infectious diseases, particularly those procedures leading to improved patient care.

Approximate Level of Support

Activity	Number	Amount
Research Grants	2	$90,578
Research Contracts*	6	$390,795
Training Grants	0	0
Fellowships	0	0
Total	8	$481,373

* Two contracts have been forward-funded with FY 1978 MIDP funds and those funds do not show here.

Program Areas

Closed Volunteer Facilities

The CSB currently supports one research contract with the University of Maryland. This contract maintains and supports The Center for Vaccine Development (CVD) where volunteers are studied before and after they are challenged with potentially transmissible infectious agents and candidate vaccines.

During the past year, the contractor performed 24 volunteer studies which were components of six more comprehensive research protocols approved by the Institutional Review Boards of the University of Maryland and NIAID. The challenge studies were distributed as follows: enteropathogenic *E. coli*, seven studies, two protocols; cholera, five studies, one protocol; parvo-like gastrointestinal virus, three studies, one protocol; H_3N_2 influenza, four studies, one protocol; H_1N_1 influenza, five studies, one protocol. Highlights of the studies in volunteers are summarized below.

A volunteer model of El Tor cholera was established to study mechanisms of immunity to El Tor and to evaluate cholera vaccines. The clinical severity of El Tor cholera, Inaba and Ogawa serotypes, was distinctly less than that previously seen with classical cholera strains of the same serotypes. Further studies also suggest that although *V. cholerae* in endemic areas may be waterborne, the contaminated water must be ingested with food in order for clinical disease to occur in the normochlorhydric host. A naturally occurring, non-

toxigenic El Tor strain isolated from sewage in Brazil was shown to have most characteristics demanded of an oral, attenuated vaccine strain; it will be tried as a vaccine in the near future. Preliminary trials with an oral, killed Inaba cholera vaccine suggested that the vaccine was efficacious; further studies are planned. A highly purified E. coli type 1 somatic pilus vaccine prepared by Dr. C.C. Brinton, Jr., University of Pittsburgh, was shown in preliminary studies to have low local reactogenicity, absent systemic toxicity, excellent immunogenicity, and efficacy (P = .04) in protecting immunized persons against challenge with the virulent homologous E. coli strain. The CVD found the ELISA for measurement of IgG cholera antitoxin in human sera to be a simple, sensitive, and reproducible test to carry out sero-epidemiologic studies. In collaboration with the Center for Disease Control they found that long-lived cholera antitoxin is stimulated by natural infection with V. cholerae or enterotoxigenic E. coli. In collaboration with the NIAID Laboratory of Infectious Diseases, studies were carried out with small particle (27 nm) agents of gastroenteritis to measure serum and intestinal antibody and resistance to rechallenge. Stools containing the 27 nm Norwalk agent and Hawaii agent were collected as sources of virus for biophysical studies; results are pending. One of twenty-one volunteers inoculated intranasally with $10^{7.0}$ $TCID_{50}$ of A/Alaska/6/77-ts-1-A_2 (H_3N_2) attenuated influenza vaccine strain developed systemic illness. No reactions occurred in 18 volunteers inoculated with A/Alaska/6/77 CR29 (H_3N_2) cold-adapted attenuated vaccine strain. Two of 12 volunteers developed systemic illness after A/Hong Kong/123/77 ts-A_2 (H_1N_1) attenuated influenza vaccine, while one of 12 volunteers inoculated with the cold-adapted strain developed coryza but no systemic illness.

Collaborative Clinical Trials With Antibiotic Therapy

The major aim of this program is to sponsor collaborative clinical trials designed to improve medical care of patients with bacterial and mycotic infections. Approved antibiotics, or promising new drugs that lack the economic potential to justify testing by the pharmaceutical industry, are evaluated in patients with selected infections.

During FY '79 a contract was initiated with the University of Alabama to study ways to improve therapy of cryptococcal meningitis. The first part of this three part protocol includes a controlled clinical trial by 14 collaborating institutions to determine the shortest treatment regimen utilizing Amphotericin B (AMB) and 5-fluorocytosine (5-FC) in combination. Four and six week treatment regimens are being compared. The Collaborative Cryptococcal Study Group during the first year has made limited progress in patient enrollment, but has nevertheless achieved balanced randomization into the two treatment groups. Progress was slower than projected because of the unexpectedly low occurrence of the disease this year. It is anticipated this can be offset in future by more aggressive publicity and a recruitment campaign among the medical community. The original plan for part 2 of the protocol was designed to evaluate the toxicology of Amphotericin B methyl ester (AME) in patients with selected disseminated mycotic infections. Preliminary clinical and laboratory data from another open trial suggested that the long-term administration of AME may be associated with the induction of neurological abberrations and various gradations of a diffuse leucoencephalopathy. Pending a thorough

review and determination by the FDA, an indefinite moratorium has been placed on the clinical administration of this antimycotic agent. Before the moratorium, the University of Alabama subcontracted with the Kresge Hearing Institute, University of Oregon to conduct ototoxicity studies with AME in laboratory animals. This study may help predict the ototoxicity and neurotoxicity of AME and other methylated polyene-like antifungal agents now under development.

Following a contractor's meeting of the Mycotic Disease Collaborative Group in June, 1979, we decided to replace AME and conduct phase II studies with ketoconazole (R41,400), a new, oral imidazole derivative with broad-spectrum antimycotic activity and low toxicity developed by Janssen Pharmaceutical of Belgium. The Collaborative Group will evaluate oral ketoconazole for safety and effectiveness in the treatment of the following group of systemic mycotic diseases: chronic cavitary histoplasmosis, disseminated or localized blastomycosis, disseminated coccidioidomycosis without meningitis, disseminated sporotrichosis, and non-meningeal cryptococcosis. Efficacy studies of ketoconazole in animal models of cryptococcal meningitis will also be done.

In response to an RFP, issued during FY '79, a new contract was awarded to the University of Illinois to perform a placebo-controlled, double-blind evaluation of the efficacy of penicillin in the prevention of early onset Group B Streptococcal (GBS) sepsis in the newborn. In addition, the study is designed to determine if penicillin prophylaxis influences the rate of late onset GBS disease, increases or decreases the likelihood of later penicillin hypersensitivity, and causes the emergence of penicillin resistant GBS or other penicillin resistant organisms. It is anticipated that this study will require at least three years and 20,000 mother-infant pairs before conclusive data will be available to demonstrate efficacy of the prophylactic regimen.

Based on recommendations from the NIAID Symposium on the Impact of Infections on Medical Care in the U.S., held May 30 - 31, 1978, Staff prepared and issued an RFP during FY '79 entitled "Improved Therapy of Endocarditis Due to Viridans Streptococci." Response to this RFP was limited to one technical proposal. During the primary review process it was determined that a controlled clinical trial was not feasible, because a properly designed trial would take too long (seven-ten years) and the resulting costs would be excessive. With this information, a determination was made by Staff to cancel the RFP and not award a contract for the trial. Subsequently, plans were developed to hold a Consensus Development Conference on the optimum therapy of penicillin-sensitive, non-enterococcal endocarditis. The conference will be co-sponsored by the NIAID, NHLBI, the NIH Office of the Medical Applications of Research, and the American Heart Association.

Rapid Viral Diagnosis

The purpose of this program is to place greater emphasis on the rapid diagnosis of viral infections. The ultimate goal is to provide the physician with reliable tests so that a specific viral diagnosis can be made in time to help in the management of the patient. To do this, the methods must be practical, precise, reproducible and inexpensive, and simple enough to be conducted safely in the physicians' offices or in small hospital laboratories. Three contracts

were awarded for the development of such tests. A contractor at the University of California, San Diego, will further develop an immunological technique that incorporates two important features. The first is the immobilization of the virus on filter paper discs and washing using an immunofiltration manifold. This technique eliminates the need for primary capture antibody, and efficiently immobilizes infected cells and viral antigens. The second important feature is the use of purified, radiolabelled or enzyme labelled Staphylococcal protein A, which binds to the Fc portion of certain mammalian immunoglobulins thereby eliminating need for antispecies detection sera. The investigators will adapt this methodology to detect influenza viruses, respiratory syncytial virus, parainfluenza viruses, and cytomegalovirus in less than one hour. A contractor at the Harvard School of Public Health will use sensitive, solid-phase immunoassays utilizing viral antibodies covalently coupled to nylon balls or nylon powder in an enzyme-linked immunoassay. This increases the amount of antibody that can be immobilized, and lessens the problem of antibody desorption. The test is now sensitive enough to permit rapid identification of influenza virus in clinical specimens, and will be applied next to other respiratory viruses. A contractor at Johns Hopkins Hospital will develop two tests for rapid viral diagnosis. The first is an enzyme-multiplied radioimmunoassay (EMRIA) which is the standard enzyme immunoassay but uses a tritium labelled substrate. This test has been shown to detect small quantities of non-replicating cytomegalovirus antigen in clinical specimens. The enzyme-linked fluorescent assay (ELFA) using a fluorescent substrate is somewhat less sensitive than EMRIA, but it is more rapid and simpler to use. These two tests will be adapted to detect other respiratory viruses. Coordination of activities within the NIH and with the Pan American and European groups on Rapid Viral Diagnosis continues to prevent duplication of effort.

Nutrition, Infection and Immunity

The goal of this program is to promote research on the interaction of malnutrition, infection and immunity in American hospitals and in overseas populations. Since the malnourished patient is at high risk for infection, this goal is consistent with the overall mission of the CSB which is to encourage research leading to improved patient care.

In FY '79 the NIAID program in Nutrition, Infection and Immunity consisted of 12 grants which were monitored by at least four project officers in the IAID and MID programs. The research focused on four different areas; the modulating effect of specific nutrients on immune function, mechanisms of food allergies and immune response to ingested antigens, the interaction of nutrition and infection in the tropical environment and in American hospitals, and the modulating effect of specific nutrients on microbial virulence.

During FY '79, the Director of NIH appointed Dr. Edelman vice-chairman of the NIH Nutrition Coordinating Committee (NCC). Through this committee and in an effort to stimulate research in nutrition, Dr. Edelman helped design and initiate several new nutrition research programs. One of these new initiatives was the establishment of clinical nutrition research units (CNRU) sponsored by NIAMDD, NIA, and NCI. Infection and immunity research appeared in several approved proposals that were submitted in response to the RFA on CNRU. These units should help stimulate research in clinical nutrition, and will serve as

models of excellence for many American academic centers. Another initiative involved the establishment of institutional and individual fellowship training grants in nutrition. Seven Institutes have agreed to fund these National Research Service Awards. NIAID has agreed to co-sponsor Research Career Development Awards in clinical nutrition when the NCC develops a joint NIH announcement for these in the near future. Dr. Edelman served on ad hoc study sections to critique applications submitted to the Fogarty International Center for the support of national and international nutrition meetings.

Dr. Edelman served as chairman of an international workshop sponsored by the NCC entitled "Nutritional Support of the Patient: Research Directions for the 1980's." The participants in this two day workshop held in September, 1979, developed a set of research priorities that will be used for program planning by eight Institutes of the NIH. The workshop focused on the feeding of the sick patient by means of enteral and parenteral nutrition support. One of the six workshop panels was relevant to NIAID programs, and delt with trauma and infection. The workshop proceedings will be published by the American Journal of Clinical Nutrition.

Dr. Edelman delivered three invited lectures on "Nutrition, Infection and Immunity" at the Walter Reed General Hospital and Walter Reed Army Institute of Research. Staff of the CSB continued their collaboration with the Diet, Nutrition and Cancer program of the National Cancer Institute by helping assess the effect of hyperalimentation and improved nutrition on infections and the immune response in selected cancer patients. The final results of this study will be known early in FY 1980.

Other Activities

Mrs. Mattheis collects and reviews the necessary documentation required to file and maintain Investigational New Drug Applications (INDAs) with the Food and Drug Administration (FDA) for the Microbiology and Infectious Diseases Program. Currently 18 active INDAs are filed with the Bureau of Biologics, FDA, and four with the Bureau of Drugs, FDA. During the past year new INDAs were filed for the study of "Meningococcal Group B Type 2 Outer Membrane Protein Vaccine", "Live, Attenuated Respiratory Syncytial Virus Vaccine", "Amphotericin B Methyl Ester Aspartate", and "Topical 9-(2-hydroxyethoxymethyl) guanine." Staff support is provided for the NIAID-Clinical Research Subpanel (CRS) which reviews both intramural and contract supported clinical studies. All contract supported clinical studies are reviewed by the CRS initially and every three years thereafter, and by the NIAID-Clinical Director, in the intervening years.

The CSB and the Bureau of Biologics, FDA, co-sponsored an International Symposium on Potentiation of Immune Response to Vaccines, held at the NIH on February 20 - 21, 1979. The symposium participants discussed the influence of molecular structure on the adjuvanticity of muramyl dipeptide and other synthetic immunological adjuvants, including liposomes and polynucleotides. The mechanisms of immunoregulation by these synthetic substances, and their use as immunological probes and as potential vaccine potentiators in man was reviewed. The possibility was discussed of certain adverse side effects attending their use in man. Nevertheless, synthetic adjuvants, including 800 variants of the muramyl dipeptide molecule alone, are emerging as exciting new substances with

many basic and applied applications.

Dr. Edelman served on the following committees of other Federal agencies: 1) NIH liaison member on the Infection Control Committee of the Veteran's Administration, 2) the Human Ethics Committees (Institutional Review Boards) of the Walter Reed Army Institute of Research and 3) the Frederick Cancer Research Center. As ex-officio voting member of the National Commission on Digestive Diseases, Dr. Edelman participated in the natural demise of the Commission when it officially submitted its Report to Congress on January 31, 1979.

Dr. Horton served as liaison member of the NIH Coordinating Committee on Cystic Fibrosis and participated at the National Scientific Working Conference on Cystic Fibrosis, March 4 - 7, 1979, South Padre Island, Texas. This working conference, co-sponsored by the Cystic Fibrosis Foundation, NIAID, NIAMDD,and NICHD was highly productive, and provided for an excellent exchange of scientific information among multiple disciplines. It identified future research opportunities with guidelines for implementing these activities. A report on this conference will be prepared and distributed by the Cystic Fibrosis Foundation.

DEVELOPMENT AND APPLICATIONS BRANCH

It is the primary objective of the Development and Applications Branch to translate new information derived from basic research into methodologies appropriate for the control or prevention of designated infectious diseases in humans. Fundamental and applied research activities supported by the Branch include: identification of important infectious disease problems with potential for control or prevention through immunization or utilization of antivirals, development of appropriate vaccines, antivirals, and other control measures, design and support of appropriate clinical trials for evaluation of control measures, and basic research in related areas.

Approximate Level of Support

Activity	Number	Amount
Vaccine Evaluation Centers		
Research Contracts	5	$ 1,218,351
Influenza		
Research Contracts	11	2,103,956
Interagency Agreements	2	51,138
Research Grants	24	2,192,305
Fellowships	2	25,237
Young Investigator Awards	2	83,812
Respiratory Diseases		
Research Contracts	1	0
Interinstitute Agreements	1	47,872
Research Grants	4	264,492
Hepatitis		
Research Contracts	7	1,160,711
Interagency Agreements	2	157,497
Research Grants	8	877,193
Career Awards	2	86,665
Young Investigator Awards	1	36,195
Antiviral Substances		
Research Contracts	13	2,659,248
Research Grants	29	2,337,501
Career Awards	2	71,432
Fellowships	2	20,877
Training Grants	1	85,688

Approximate Level of Support (cont.)

Activity	Number	Amount
Bacterial Vaccines		
Research Contracts	13	$ 816,197
Interagency Agreements	1	10,821
Research Grants	18	1,076,870
Career Awards	2	50,551
Fellowships	2	32,400
Enteric Diseases		
Research Contracts	7	762,565
Interagency Agreements	1	33,750
Research Grants	35	2,105,048
Career Awards	1	16,200
Fellowships	1	14,200
Program Projects	1	355,100
Training	1	36,504
Viral Vaccines		
Research Grants	7	548,327
Conference Support	1	29,750
Totals	209	$19,368,453

Program Summary

The initial portion of this fiscal year (through March) was heavily involved in completing studies directed at the control of influenza A/USSR/77 virus, the most recent strain shift. Clinical studies to determine vaccine safety, reactogenicity, and antigenicity, as well as its sensitivity to amantadine, were performed. Two other areas of concentrated effort in the influenza program were further development and study of attenuated vaccine candidates and determination of the role of amantadine in influenza disease control. A meeting was held in February at the Influenza Research Center to compare results with Soviet scientists on respective experiences with amantadine and rimantadine. Based on results from investigations supported by this program, there is a revived interest in amantadine. A consensus development exercise on the role of amantadine in the prevention and treatment of influenza is currently being planned.

Following the advice of a Workshop on the Development of Antivirals, the Antiviral Substances Program (ASP) developed a request for proposals for the development of targeted antivirals, and a contract has been awarded. Having determined that adenine arabinoside monophosphate (ara-AMP) was not effective as a topical drug against herpes labialis, clinical studies were initiated using acyclovir which appears to hold greater promise

based on animal studies. Protocols have also been developed to evaluate systemic ara-AMP against herpes infections, and phase 1 studies are proceeding with acyclovir. If these latter studies prove satisfactory, this drug will be considered for clinical efficacy studies. Final determination on the establishment of interferon standards and their distribution has been made with WHO. This is particularly important based on the renewed efforts in clinical application of interferon against infectious diseases and cancer. Through the efforts of this program, the first complete summary of all clinical studies with interferon was published (Journal of Infectious Diseases, 139:109-123, 1979). One of the most exciting findings of the ASP during the past year was the apparent efficacy of adenine arabinoside in neonatal herpes. Although the data are still being analyzed, the results appear similar to the efficacy of this drug against herpes encephalitis.

A new initiative for the viral vaccine program was the release of a request for proposals to evaluate candidate varicella vaccines. Herpes varicellæ produces a highly contagious but mild disease (chicken pox) in healthy children; however, it causes severe disease with increased morbidity and even death in immunosuppressed children. At a Workshop on Experimental Herpesvirus Vaccines, it became clear that varicella vaccines with clinical potential were available and definitive clinical studies should be performed.

Two other new areas in the Branch's vaccine program are based in the Bacterial Vaccine Program. The current Rocky Mountain Spotted Fever vaccine was withdrawn from the market based on lack of efficacy; this created a need for accelerated work with a new vaccine candidate developed by USAMRIID. Studies are underway on epidemiology and animal studies in anticipation of future clinical studies. An International Symposium on Pertussis, principally co-sponsored by this program and the BoB, raised many questions concerning the pertussis vaccine and considerations on possible improvement of this vaccine are underway. Another major effort of this program was the release of a request for applications for studies on infant (less than two years of age) infections. This is an offshoot of the studies on meningitis vaccines. In these studies, it was shown that bacterial polysaccharide antigens were not as effective in this target population as desired.

The Hepatitis Program is completing phase 1 and 2 studies prior to efficacy studies of the hepatitis B (HBV) vaccine. It has been determined that the HBV vaccine containing alum adjuvant is preferable to the aqueous preparation. The alum vaccine will be used in the proposed hemodialysis center study. Clinical studies to evaluate the possible effect of hepatitis B immune globulin to prevent transmission from hepatitis antigen positive mothers to newborns are underway. The woodchuck appears to be a useful animal model for hepatitis pathogenesis studies; attempts to develop this model are underway.

Epidemiologic studies on diarrheal diseases initiated in FY77 are beginning to yield interesting results. Trends related to age, crowding, and etiological agents are emerging. Studies with cholera vaccine in endemic areas continue. Currently two vaccines (provided by the Wellcome Foundation), an alum precipitated whole cholera cell and a purified toxoid complexed with aluminum hydroxide, are being tested individually and in combination. A major breakthrough

in the treatment of cholera has been the use of chlorpromazine in reducing fluid loss in these patients. Of considerable interest was the finding of cholera vibrios in U.S. estuaries. This is being followed with great care.

In FY79 the DAB was involved in the organization and conduct of six major meetings: International Symposium on Pertussis, Workshop on Collaborative Pneumococcal Vaccine Studies, Workshop on Hepatitis B Vaccine, Experimental Herpesvirus Vaccine Workshop, Conference on Cholera, and Workshop on Influenza B Viruses. Summaries on each of these meetings, with the exception of the Pneumoccal Vaccine meeting, will be published.

Research Highlights

Influenza

The Influenza Program has experienced another very active year. As with the previous year, the major development in influenza has been the reappearance of viruses which are antigenically very closely related to those which produced epidemic disease during the 1947-1957 period. The prototype of this new era was the A/USSR/77 (H1N1) virus which has been shown to be, during the last year, antigenically and biochemically quite similar to the A/FLW/50 (H1N1) strain which circulated in 1950. Recent data suggest that the difference between A/USSR/77 (H1N1) and A/FLW/50 (H1N1), a 1950 strain, may be only eight base pairs in the nucleic acid. Although it was widely anticipated that a change in the predominant type could be expected later in the 1970s, the reemergence of the H1N1 strains was totally unexpected. These new variants have been very restricted in their ability to produce disease and virtually all cases have been seen in individuals born after 1955 or age 0 to 24 years. The new variants also appear to have supplanted the earlier H3N2 strains as the predominant virus. Only scattered isolates of H3N2 viruses have been reported during the last winter, none from the United States.

During the last year, the Influenza Program completed the evaluation of the A/USSR/78 inactivated vaccines. Over 2100 subjects participated, including approximately 1300 under the age of 24 years and 300 subjects considered at high risk to influenza. Final reports of these studies are being completed and will be submitted for publication during this fiscal year. Field trials of three other vaccines under development were also conducted. The two attenuated vaccines were subjected to extensive tests. These include the preliminary clinical evaluation of these vaccines in approximately 200 subjects under closed conditions, 200 subjects to determine transmissibility, and over 2100 subjects during an open-field trial at Texas A & M University. However, the test population did not experience a large enough natural challenge, and it will not be possible to determine vaccine efficacy. The vaccines did not produce any significant reactivity. The subjects will be followed to determine their antibody status and their clinical experience after natural challenge with related H1N1 viruses. In addition, some of these vaccinees will be challenged by related and homologous viruses to obtain information on the duration of immunity induced by these vaccines. The third vaccine, a purified subunit vaccine, was also tested and a formulation was developed which induced excellent antibody response in subjects with HAI titers less than 1:8.

The utility of amantadine as a prophylactic and therapeutic agent in influenza was clearly demonstrated last year. Studies will continue to explore the use of amantadine in high-risk patients and in the therapy for pneumonia. Rimantadine will also be studied. In collaboration with the Antiviral Substances Program, pilot studies on the possible synergistic effect of rimantadine and ribavirin have been started. Soviet investigators had earlier reported a synergistic effect with these drugs. Studies using a plaque-reduction assay have shown that these drugs are remarkably effective when used in combination.

The results of the studies presented above should contribute directly to the control of influenza. The basic scientists supported by the NIAID and working with influenza viruses have continued to make significant contributions to our understanding of myxo and paramyxoviruses. Recent data suggest the presence of a ninth viral protein. This same investigator has also shown that antibody to the F protein of Sendai virus inhibits syncytia formation. This may have important implications with regard to infections produced by related viruses, such as respiratory syncytial virus infection, and to the possible design of vaccines for these viruses. Other investigators have shown that certain genes in influenza are not randomly reassorted during recombination. In fact, two pairs appear to be "linked." Since these pairs involve the RNA polymerase genes, this observation may be quite important in attenuated vaccine development. This group has also found that resistance to amantadine is linked to the M protein. Persistent viral infections of influenza viruses have also been obtained independently by two investigators. The significance of these observations is not yet clear, but these in vitro systems should enable a variety of studies to examine the biochemical aspects of viral interference and viral persistence.

The supplemental funds provided to the Influenza Program during the swine flu and Russian flu era have been expended, and there is a resulting reduction in directed program activities. There are currently 12 contracts compared with 15 last year. This includes one new contract awarded this year to identify alternate cell substrates for diagnosis and cultivation of influenza virus. Among the projects which were terminated was the influenza surveillance network which had effectively served as a sentinel system since 1976. In contrast, the number of investigator-initiated projects has dramatically increased with the addition of four grants to a total of 24, two young investigator awards and two fellowships.

Respiratory Diseases

The magnitude of infection and illness from parainfluenza viruses types 1, 2, and 3, and respiratory syncytial virus (RSV) is difficult to document, but estimates from several epidemiologic studies indicate that these viruses cause severe morbidity in pediatric populations. Of all severe lower respiratory diseases in infants and young children, perhaps 40 percent could be prevented if effective vaccines against these agents were available and used.

Efforts to develop an attenuated respiratory syncytial virus vaccine have produced a temperature sensitive mutant that has been satisfactorily tested in animal models and human adults. Studies in children are underway. The testing of the live vaccine developed by Merck Sharp and Dohme has been completed in animal models. These studies were not promising, and results from an efficacy trial (R. Belshe, personal communication) indicate no benefit. The NIAID does not plan any further tests with this product.

Studies on the epidemiology and pathogenesis of RSV and parainfluenza infections continue at Baylor University and the University of Rochester. Longitudinal family studies have shown a positive correlation between serious RSV disease and age at time of first infection. These studies have also shown that maternal antibodies are protective in the first months of life. Several factors such as crowding of living quarters, hygienic practices and number of persons having regular contact with the infants, may be related to the likelihood of developing disease. Month and year of birth are other obvious variables.

Viral Hepatitis

The goal of the Hepatitis Program is the control of hepatitis A (HAV), B (HBV), and non A-non B (NANB) infections. Precise data on morbidity, mortality, and economic burden to the country are difficult to obtain; however, as investigations continue on the etiology, epidemiology, and long term sequelae of viral hepatitis, it has become apparent that many cases have been misdiagnosed or underreported. In 1977, approximately 56,000 cases of viral hepatitis were reported to the CDC. Allowing for misdiagnosis and underreporting, it can be estimated that 560,000 cases of viral hepatitis occurred in the U.S. in 1977, with a distribution of approximately 50 percent HBV, 25 percent HAV, and 25 percent NANB. Estimates of death from HBV range from 1,500-3,000 (approximately 1 percent fatality rate) per year. Costs incurred from hospitalization, professional care, laboratory work, and days lost from employment are estimated to exceed $600 million annually.

Type B hepatitis has an incubation period of 60-180 days, and may exhibit an acute and chronic phase. Type A hepatitis has a shorter incubation period of 20-40 days and does not exhibit a chronic infection. NANB hepatitis, with an incubation period similar to HBV, is responsible for approximately 90 percent of the post-transfusion hepatitis remaining in the U.S. Although the virus(es) responsible for this type of hepatitis has not been identified, the disease has been successfully transmitted to chimpanzees.

Because of its more serious clinical manifestations, type B is a disease of major public health importance in the U.S. However, the global impact of the disease is more significant when 176,000,000 chronic carriers remain as a reservoir for infection. In addition to serving as reservoirs of virus, a close association of chronic HBV and primary hepatocellular carcinoma (PHC) has been demonstrated. The role of the HBV in PHC remains to be defined, but cause-and-effect will be difficult to ascertain.

During the past year, efforts have continued in evaluating experimental hepatitis B vaccines in humans. As noted last year, aqueous formalin-inactivated vaccines prepared by the NIAID were shown to be non-reactogenic and non-infectious in volunteers. To date, 16 volunteers have been immunized with the adw vaccine and, although most have developed humoral antibody, the long delay between boosters and appearance of antibodies indicates that these vaccines would not be practical for clinical use.

Further testing of the ayw vaccine has not proceeded since one of the five volunteers participating in the initial safety tests developed NANB hepatitis. At the present time, evidence suggests that the vaccine was not infectious, but further definitive analyses must await the development of serologic markers for NANB virus.

Independently, Merck Sharp and Dohme has been exploring the development of a type B hepatitis vaccine. Their procedure, similar to the NIAID procedure, employs isopycnic banding and rate zonal centrifugation of the 22 nm surface antigen with subsequent inactivation with formalin. They have evaluated two types of vaccines in chimpanzees and humans -- aqueous and adjuvanted (aluminum hydroxide). Their data with aqueous vaccine are comparable to the NIAID data, safe, but not adequately immunogenic. However, the alum absorbed vaccine did appear highly immunogenic with approximately 65 percent of the volunteers developing antibody three and one-half months following a two dose regimen and 90 to 95 percent presenting antibody following a booster at six months. Merck has offered their vaccine for testing for efficacy by the NIAID. The independent evaluation by the NIAID of a potential vaccine that is earmarked for licensure will lend additional credence to data generated in efficacy trials, and represents the testing of vaccine in a population that is at extremely "high risk" for B virus infection and disease, i.e., hemodialysis patients and staff.

In preparation for an HBV efficacy trial, the New York Blood Center has been documenting the incidence of viral hepatitis in patients and staff within selected hemodialysis centers. The results of this study show that the current attack rates appear low compared with rates observed in the early 1970s, yet are still 10-20 times higher than in other groups of hospitalized patients and staff. Based on incidence of HBV infection, availability of the test population, and ability to give informed consent, this population represents a feasible group for vaccine efficacy. The efficacy study will start approximately September, 1979, and will be a randomized, double-blind placebo-controlled trial. It is estimated that 1,000 patients and 900 staff will be randomized into the trial in a two year period. The vaccine to be employed will be a monovalent ayw, alum-absorbed preparation provided by Merck Sharp and Dohme. It is expected that efficacy data should become available in the Fall-Winter, 1982.

Another approach to prevention and control of HBV infection and sequelae is to interrupt maternal-fetal transmission. Neonatally acquired infection is of major magnitude in parts of Africa and the Far East. For example, 85 percent of newborns born of HBsAg+ HBeAg+ mothers in Taiwan become infected within the first six months of life. The NIAID and BoB/FDA are collaboratively

supporting a study to evaluate Hepatitis B Immune Globulin (HBIG) for interruption of this transmission route. During the past year, 115 babies have been randomized into the double-blind, placebo-controlled trial. Seventy more babies will be entered into the study prior to January, 1980. Follow-up of the babies for one year will be required before the data can be interpreted. Thus far, there have been no adverse reactions to the HBIG, but since the code has not been broken, there are no results available.

The virus of hepatitis B has not been cultivated in vitro. Attempts to develop continuous hepatocyte cultures of human and chimpanzee origin have been unsuccessful, and contractual support for this activity is terminating this year. Hopefully, investigator initiated approaches to this requirement will be forthcoming. Successful cultivation of hepatitis A virus was reported during the past year by investigators at Merck Sharp and Dohme.

Exciting observations have been made on a virus isolated from woodchucks (Marmota monax). This virus has many characteristics in common with human HBV, including morphology, DNA of similar size and structure, presence of endogenous DNA polymerase, and association with hepatitis and PHC. Infection of woodchucks with this virus may represent the best and only animal model for hepatitis and its relation to PHC, and studies are now underway to better characterize the animal-virus system. A continuing problem will be the availability of woodchucks, an animal that hibernates from late fall to early spring. It is clear that research will be severely hampered until young animals of known serologic status are readily available to investigators.

Recent breakthroughs in DNA recombinant research have the potential for rapidly changing approaches to molecular aspects of HBV. At least three laboratories have reported the successful cloning of the HBV genome in Escherichia coli, with one laboratory reporting expression of HBcAg. The ease of which this technology has been applied to HBV bodes well for developing nucleic acid probes for studies of viral persistence and hepatoma and, perhaps, the elaboration of HBsAg or its component polypeptides as a source of future vaccine antigen. An NIAID grantee, Dr. William Robinson, Stanford University, is a pioneer in this area of investigation.

The use of antiviral drugs in eradication of the HBV carrier state is also being assessed and is described in the Antiviral Substances Program section.

Antiviral Substances

Virus diseases are a leading cause of morbidity and mortality throughout the world, and the use of antivirals is an important means of treating and preventing these illnesses. Although development of host resistance remains the first line of defense against viral diseases, there are many viral diseases for which vaccines are not available or cannot feasibly be given to all people at risk. Thus, chemotherapeutic agents with low toxicity and high efficacy are needed.

Herpes infections are a major cause of disease with the symptoms varying from mild labialis infection to fatal encephalitis. Carefully controlled clinical trials were supported to determine the efficacy of adenine arabinoside (ara-A, Vidarabine) for treatment of herpes encephalitis, and the use of this drug reduced the mortality from 70 percent to 28 percent. The results from these trials led to the licensure of adenine arabinoside for herpes encephalitis in October, 1978. The use of adenine arabinoside for other types of herpes infections is still under study, including studies with herpes zoster and neonatal herpes. Preliminary results indicate that adenine arabinoside will also have some beneficial effect in these diseases. The code for the neonatal herpes study was recently broken, and preliminary analysis of the data indicates a reduction in mortality of approximately 40 percent.

Systemic herpes infections can cause devastating illnesses and a wide range of antivirals is needed to treat these diseases. Adenine arabinoside is a difficult drug to administer because of its low solubility in water. Better antivirals should be developed. Animal model studies supported by the Antiviral Substances Program have demonstrated that adenine arabinoside monophosphate (ara-AMP) and 9-(2-hydroxyethoxymethyl)guanine (acyclovir, ACV) also hold promise for treatment of systemic herpes disease. These drugs are being considered as candidates for evaluation in future clinical studies against serious herpes infections in protocols similar to those utilizing ara-A.

In addition to systemic herpes infections, topical herpes infections are a major cause of disease in the United States, with type two herpes being the second most frequent genital infection in the United States, and type one herpes (herpes labialis) causing recurrent infections in over 30 percent of the adult population. Work to define the natural history of herpes labialis has been supported by the Program. This work led to a subsequent clinical trial which demonstrated that ara-AMP was not effective as a topical treatment. During 1979, a double-blind clinical trial to evaluate the effectiveness of topical application of ACV for herpes labialis was started. These studies are also designed to evaluate the general use of ACV in humans, including analysis of any potential antiviral resistance to the drug and drug absorption through the skin.

Interferon, a broad-spectrum antiviral, is a candidate antiviral for both RNA and DNA virus infections. Through grant sponsored research, we are learning more about the mechanism of action and the antigenic and biochemical nature of interferon. There are three types of interferon: fibroblast and leukocyte (type one interferons) and immune interferon (type two interferon). Much of our understanding on the nature of interferon has come about through the use of NIAID reference interferon preparations, which have allowed the comparison of results from laboratories throughout the world. During the past year, the World Health Organization has designated the NIAID reference reagents for human fibroblast, rabbit, and mouse interferons as international standards. The use of NIAID antisera to human leukocyte, human fibroblast, and mouse interferons has also enabled investigators to identify specific biochemical events brought about by this antiviral, and has played an important

part in developing better methodologies for its purification. A number of studies on purification have been initiated, and considerable headway has been made in the characterization of this elusive moiety.

Human leukocyte interferon is being evaluated in clinical trials for the treatment and prevention of herpes, hepatitis B and respiratory viral infections Recent clinical trial data indicate that interferon can reduce the reactivation of herpes infection after cranial nerve surgery by 43 percent. The total incidence of virus shedding was reduced from 41 percent in placebo patients to 8 percent in interferon patients. These studies are continuing to determine optimal dose schedules. Interferon has also been shown to be efficacious in the treatment of herpes zoster infections in the immunosuppressed patient. When administered in combination with antihistamines, it appears to hold promise for treatment of respiratory viral infections.

Currently, there are over 175 million carriers of hepatitis B for which there are no satisfactory therapies. The Antiviral Substances Program is conducting studies to determine optimal regimens for modifying or eliminating the carrier state. Recent results suggest that the best treatment for hepatitis B will be combination therapy with interferon and another antiviral, such as adenine arabinoside.

Since 1969, the Antiviral Substances Program has been the primary source of funding and support for interferon studies in the United States, and these studies have led to an understanding of the potential of interferon in the clinic. Based on the results from this Program, the National Cancer Institute and American Cancer Society have initiated carefully controlled clinical trials with interferon in a variety of cancers. Cooperation among interferon researchers continues to be an important objective of the Program.

A critical part of the effort to develop clinically effective antivirals has been the establishment of a series of animal-viral disease models which closely mimic some common viral diseases of man. During the past year, the effectiveness of acyclovir against herpes infections, including genital and systemic infections, has been demonstrated in these animal models. A new antiviral, phosphonoformate, is being studied to determine efficacy and relative toxicity. These animal studies are important in determining optimal dose schedules and routes of administration, information necessary for planning successful clinical trials in man.

A new initiative to develop targeted antivirals was started by the Program during 1979. Based on our knowledge of the nucleic acid sequences in virus genes, antivirals will be developed which will specifically interact with the nucleic acid without interfering with host cell metabolism. Targeted antiviral development is a field of the future and will be supported by both the grant and contract mechanisms.

Bacterial Vaccines

Pneumococcal Diseases: Studies sponsored by the NIAID during the past decade, along with studies by manufacturers, have led to the development and licensure of a 14-valent pneumococcal polysaccharide vaccine (Merck Sharp & Dohme). It

is expected that a second manufacturer (Lederle) will have a licensed product by the Fall of 1979.

It is currently recommended that pneumococcal vaccine be used in those individuals over two years of age considered at high risk for pneumococcal infections. There are a number of disease conditions which predispose to pneumococcal infections; however, there are little data on the immunogenicity and efficacy of pneumococcal vaccine relating specifically to these populations. The Bacterial Vaccines Program has developed a collaborative effort through which pneumococcal vaccines are being studied in patients with the following conditions: sickle cell disease, splenectomy, bone marrow transplantation; Hodgkin's disease, renal transplantation, chronic dialysis, lymphoma, myeloma, diabetes, lupus, lymphoblastic leukemia, alcoholic cirrhosis and chronic obstructive pulmonary disease. There are approximately 25 studies underway in various stages of completion. Although no direct funding is being given to the investigators by the NIAID, these studies are being coordinated by staff and all antibody assays are being done through the contract-supported serology laboratory at SUNY, Downstate Medical Center. Although these studies have not been completed, some important information is already evident: (1) Sickle cell and splenectomy patients appear to respond well to the vaccine; (2) Hodgkin's patients who have undergone splenectomy and chemotherapy respond poorly to vaccine; however, Hodgkin's patients given vaccine prior to therapy appear to respond normally; (3) antibody response is diminished in renal dialysis patients; and (4) simultaneous administration of pneumococcal and influenza vaccines does not have a negative effect on response to the individual antigens. When the collaborative studies are completed, they should allow for specific recommendations on the use of pneumococcal vaccine in various patient populations.

Pneumococci are estimated to be responsible for 40 to 50 percent of acute otitis media in children. This is an important childhood disease which often causes hearing disorders and subsequent learning disabilities. Almost every child will have at least one bout of otitis media; 20 to 30 percent will have six or more occurrences.

A study of the value of pneumococcal polysaccharide vaccines in preventing otitis media was initiated in 1975. Two double-blind efficacy trials of an octavalent vaccine for the prevention of disease in children who have had previous middle ear infections are now underway. The code has not been broken on these studies; therefore, no data on efficacy are available at the present time. Data from one of the trials (Huntsville Hospital) will be analyzed in the Summer of 1979 and the other trial (Boston City Hospital) will be completed in the Spring of 1980. A third project is designed to establish the optimum dose and booster regimen for maximum antibody response of infants and young children. Certain polysaccharides (i.e., type 3) are good immunogens in infants when given in one dose. Some cause measurable responses when given in two doses (i.e., types 7 and 18), while most of the others are poorly immunogenic in infants with any dose regimen.

To better understand the pathogenesis and immunology of pneumococcal otitis media and the efficacy of pneumococcal polysaccharide vaccine in preventing

experimentally-induced middle ear disease, an animal model system has been developed. Utilizing this chinchilla model, investigators have been able to correlate vaccination with protection from disease, even in cases in which there has been no measurable serum antibody response to the polysaccharides. These data suggest that pneumococcal vaccines may prevent some otitis media in young infants in which serum antibody response is poor.

Meningitis: It is estimated that approximately 20,000 cases of bacterial meningitis occur in the U.S. each year, most in young children and infants. A pure polysaccharide vaccine for H. influenzae type b, developed and tested under our program, has been shown ineffective in children under 18 months of age. Because most of the H. influenzae meningitis occurs in this younger age group, program efforts are now directed toward developing vaccines effective in infants. For the past few years, considerable laboratory and clinical effort has been concentrated on a new H. influenzae complex vaccine. This preparation is a complex of polyribose phosphate (PRP) and cell wall material, primarily polypeptides and lipopolysaccharide. Designated PRPc, this material showed promise in animal studies. Adult human studies have shown the new vaccine to be potentially a better antigen; however, it is considerably more reactive than pure PRP and efforts to significantly reduce the toxicity have met with limited success. Plans are being made to continue studies of this in children; however, alternate vaccine development approaches are also being followed. The alternate approaches include: (1) extraction and characterization of outer membrane proteins from H. influenzae to determine their role in disease and their potential as immunogens; (2) preparation of a complex of PRP with a pure protein such as the diptheria toxoid; (3) preparations of complexes of highly immunogenic bacterial polysaccharides with determinant groups of PRP; and (4) preparation of very high molecular weight molecules of PRP.

In addition to efforts to improve the infant's response to bacterial meningitis vaccines, protection of the neonate and young infant by hyperimmunization of the mother is being attempted. This is being done in humans by giving H. influenzae PRP to women who plan pregnancies and to a control group of women who do not plan to become pregnant. Serum samples from mothers and children will be studied to determine if the level of infant antibody to H. influenzae can be increased during the crucial first few months of life. Hyperimmunization of the mother is also being studied in primates; pregnant females will be immunized to determine the possible harmful effects of vaccine administration during pregnancy as well as to follow antibody levels in the mother and offspring.

Group A and C meningococcal polysaccharide vaccines have been licensed for use in adults and older children. The group A vaccine has been shown to be effective in children down to three months of age in an efficacy trial in Finland sponsored by the NIAID. Group C meningococcal polysaccharide vaccine has been shown to be poorly immunogenic in very young children. A capsular polysaccharide extracted from a variant group C strain has been prepared at Rockefeller University. This new material has a slightly different chemical structure from the licensed group C vaccine, but it is antigenically cross-reactive. Through the NIAID program, this variant C vaccine has been shown superior to the licensed material

in adults and in children over two years of age. Studies in infants will begin in the immediate future.

During the course of the Finnish trial of the efficacy of group A meningococcal vaccine in children (mentioned above), it was discovered that persistence of antibody and the importance of the booster regimen on persistence of antibody appear to be age related. The Finnish study is being continued to further study persistence of antibody as a function of the type of dose and booster regimen used.

Group B meningococcal polysaccharide is non-antigenic in humans. A protein antigen vaccine candidate, from the outer membrane layer of the organism, has been developed by the BoB. Phase I safety and antigenicity tests in adults showed reactions to the vaccines to be acceptable, but antibody responses were somewhat disappointing. Plans are underway, however, for studies in teenage children and children over age 2. A second cell envelope protein antigen prepared at the Naval Medical Research Institute will be tested in adults in the near future.

Rocky Mountain Spotted Fever: The presently licensed vaccine for RMSF is not considered effective and is no longer commercially available. A new inactivated, whole-cell vaccine has been developed at the U.S. Army Research Institute for Infectious Diseases (USAMRIID). Because of our mutual interest, the NIAID has assisted with support of phase I clinical trials of the vaccine in adults at the USAMRIID facilities at Fort Detrick. The vaccine appears to be superior to the commercial material in these studies and additional clinical studies may be sponsored by NIAID. Three new contracts have been awarded this fiscal year for additional animal model studies of RMSF and the USAMRIID vaccine and for studies of the epidemiology of RMSF.

Enteric Diseases

The Enteric Disease Program of the NIAID is concerned with reducing the morbidity and mortality of infectious diarrheas in this country and around the world. This includes viruses, bacteria and their products, and the unicellular parasites. The Program is seeking to define the scope of the diarrheal problem in infants, children, and adults, to identify the etiologic agents and elucidate their pathogenesis and to gain knowledge for developing practical means of control.

The Institute funded three enteric disease study centers in FY 1977 (D. C. Children's Research Hospital, The University of Michigan, and the University of Texas in Houston) for epidemiological studies leading to finding methods of controlling diarrheal diseases. Several trends are beginning to emerge: (1) Younger children have more diarrhea episodes than older children, i.e., 57.4 visits to the doctor's office/100 persons/year in the 0-4 year olds; 13.9 visits to the doctor's office/100 persons/year in the over 20 year olds in a small town and rural population and 1.7 episodes of diarrhea in the first 12 months of life in an urban area and approximately 0.6 episodes in the second 12 months. (2) Overcrowding of children in day care centers produces a higher attack rate of diarrhea, even in the more affluent populations. There were several instances of secondary cases among family members. Three of five outbreaks were associated with Shigella infections and two were associated

with both rotavirus and Giardia infections. (3) Rotavirus is a common cause of infant diarrheas with a peak attack rate in the winter as high as 71 percent during a one month period. (4) Some families (16 percent) with infants did not report any episodes of diarrhea in the first 12 months of life; about the same number (15 percent) reported two or more episodes of diarrhea. (5) Type 2 rotavirus was detected about three times more often than type 1. (6) There is a suggestion that uncultivatable adenoviruses play a role in acute diarrheal diseases.

The seventh world pandemic of cholera continues into its 18th year. There were 40 countries reporting cholera in 1978 as compared to 34 in 1977. This includes the United States where the first multicase outbreak of cholera since 1911 occurred in Louisiana in August. Crabs were implicated as the source, with their contamination coming from the environment. The Institute program on cholera is part of the U.S.-Japan Cooperative Medical Science Program. Cholera studies remain important per se, but they also serve as an excellent model for studies on enterotoxin mediated diarrheas, including studies of the local immune response.

The intensive activity in cholera research has produced the following: (1) Two laboratory-modified live vaccines for testing in volunteers; (2) Two isolates from the environment identified as candidates for a modified live vaccine; (3) Evidence that cholera toxin produces long lasting immunity in the mucosal IgA system of dogs; (4) A flagellar vaccine which produces better protection in rabbits than licensed whole cell vaccine. This protection is enhanced 1,000-fold when combined with cholera toxoid; (5) Chlorpromazine is capable of drastically reducing fluid loss in patients suffering from cholera; and (6) Non O Group 1 cholera vibrios are present in U.S. estuaries as well as in the Ganges River delta, the perennial seat of cholera infection. These vibrios produce no detectable toxin; however, some activate adenylate cyclase in a manner similar to cholera toxin.

The treatment of cholera with fluids has been refined many times in the past decade. This past year provided a major new breakthrough in treatment when it was determined that chlorpromazine was capable of greatly reducing the fluid loss. This is the first example of a safe, practical, pharmaceutical agent with this capability. The implications are far-reaching.

The Wellcome Foundation Ltd., Beckenham, Kent, England, has provided an alum precipitated whole cell cholera vaccine, a cholera toxoid complexed with adjuvant, and the two products combined for field testing in Dacca, Bangladesh. The trial is being designed to determine the protective role of an alum precipitated whole cell vaccine and the additive or synergistic effect of the toxoid combined with the vaccine.

Studies on the interaction of cholera vibrios with the intestinal mucosa of mice indicate chemotaxis and protease (mucinase) contribute to virulence. This suggests there are other antigens which may be useful in new cholera vaccines, (e.g., mucinase) and also characteristics which, when absent, may render live mutants avirulent and thus of potential value as living oral vaccines. Vibrio ecology studies are of increasing importance with the report of cholera in

Louisiana traced to seafood. V. cholerae isolates in marine estuaries are usually the avirulent non O Group 1 vibrios. Selected isolates will be tested for virulence in volunteers. Laboratory tests to date indicate no toxin production; however, there is an indication of ability to activate adenylate cyclase as cholera toxin does. The history of cholera in Louisiana indicates that cases may recur. Similar situations may exist in other areas in this hemisphere and pose an obvious threat to places with poor sanitation. A better understanding of the role of vibrios in the environment is needed to suggest methods of control.

Considerable progress has been made in studies of disease caused by toxigenic E. coli by using techniques developed in cholera studies. Evidence continues to show that these strains are a major cause of diarrhea among travelers to developing countries and among children and adults in these countries. They occasionally cause diarrhea in the U.S., especially in newborns in crowded areas with poor sanitation. Both the heat labile (LT) and heat stable (ST) enterotoxins of E. coli have been purified. The LT has a subunit structure (A&B) very similar to cholera toxin, and they are immunologically related to A&B cholera toxin subunits. There are minor differences in both E. coli subunits compared to the cholera toxin subunits; however, the biologic activity is comparable on a weight-for-weight basis. The structure and mechanism of action of ST are being studied. Diarrhea from E. coli appears to be related to the organism's ability to attach to the intestinal mucosa. Two distinct pilus-like colonizing factors have been identified on human pathogens (CFS I and CFA II).

Antibody studies indicate there may be a protective immune response to the CFAs, and this is being pursued. It appears there are other factors of E. coli which contribute to diarrheas. One is a S. dysenteriae-like toxin which is pathogenic in rabbits. Also, some E. coli without known pathogenic entities cause diarrhea in human volunteers, indicating other virulence mechanisms. Genetic manipulation of the LT gene of E. coli may lead to the production of a strain for immunoprophylaxis.

Viral Vaccine Program

The viral vaccine program covers those areas where research should be stimulated toward the goal of an effective vaccine but which is not covered by the other specific programs of the Branch. During the past year an Experimental Herpesvirus Vaccine Workshop was co-sponsored with BoB, NCI, and the Fogarty International Center. It became clear that although attempts at vaccine development against Herpes simplex, cytomegalovirus, and varicella viruses had been made, only the varicella vaccine showed promise. Since varicella virus causes serious disease in immunosuppressed children or children with malignant diseases, an RFP to evaluate the efficacy of this candidate vaccine was released. Work with this vaccine has been initiated through a grant with the University of Texas at San Antonio for phase 1 studies. The program continues to support research to determine the antigenic structure and other characteristics of the herpes viruses in the hope of developing other more effective vaccine candidates.

The Development and Applications Branch also supports five General Vaccine Evaluation Centers which form the backbone of the Branch's efforts at control

of important disease problems. Through these groups, different populations, including all age groups, normal and "at risk," are available. These groups are utilized for the evaluation of all vaccines, viral and bacterial, and antivirals of interest to the Branch. During this past year these Centers have been overtaxed and a backlog of work has developed.

FY 79 has been a successful year with significant progress in vaccine development, licensure of adenine arabinoside for herpes encephalitis, promising results in the efficacy of ara-A for neonatal herpes, and interferon for trigeminal neuralgia, zoster, and chronic hepatitis B.

EPIDEMIOLOGY AND BIOMETRY BRANCH

The Epidemiology and Biometry Branch personnel supports the research program of the Institute through it's own projects and through collaboration with other units in the MIDP and the Institute. Collaborative arrangements included consultation in study design, evaluation of the epidemiological approach of studies, advice on management of large research data sets, analysis of research data and monitoring data from multicenter clinical trails.

Approximate Level of Support

Activity	Number	Amount
HLA and Infectious Disease Epidemiology		
Research Contracts	2	$ 52,452
Nosocomial Infectious Epidemiology		
Research Contracts	1	500,000

HLA and Infectious Disease Epidemiology

The EBB program which addresses questions about HLA and Infectious Disease Epidemiology was supported by two projects in FY79. The study of the association between HLA phenotype and recurrent herpes virus type 1 infection, which is sponsored jointly in Framingham, MA, by NIAID, The Bureau of Biologics and NHLBI, continued through the collection of clinical data and the collection of serum specimens. These data may contribute to our understanding of the relationships host factors such as HLA as circulating antibody have on a not uncommon expression of a universal infection.

A second project in this program was started in FY79 when investigators at the Immunogenetics Laboratory of the Johns Hopkins University were awarded a contract in The Influence of HLA on the Immune Response to Viral Vaccines in Humans. This study is being conducted among families of the Old Order Amish which Dr. Wilma Bias, the Principal Investigator, has characterized previously with regard to HLA. An association between any differential in the dynamics of the various cellular and humoral components of the immune response to polio, mumps, rubella or measles vaccines and HLA will be sought. Should crude associations be noted it is anticipated the extensive and reliable geneologic information and the access to family members for study may allow the investigators to identify genes separate from but closely linked to HLA genes which modify the immune response to vaccines.

Influenza

The entire EBB staff participated actively in influenza research activities in FY79. Data from the clinical trials of inactivated A/USSR vaccine was validated, organized and provided to the various investigators.

Dr. Blackwelder, Dr. David Alling of the LCI and Sir Charles Stuart-Harris, a Fogarty International Scholar, conducted an investigation of relationships among total and cardiovascular mortality and influenza activity as measured by acute respiratory mortality and influenza mortality. This is an effort to fit U.S. data to a model of these relationships reported by British workers. If U.S. data produce a sufficiently good model it is anticipated that quantitative analysis of patterns of death among the elderly will identify clearly an influenza-related portion of mortality which will allow preventive strategies to be brought to bear more efficiently on truly high-risk persons. Policy decisions relating to influenza immunization of all people over 65 require reliable estimates of morbidity and mortality, and what proportion may be prevented by various strategies. Epidemiology is central to thorough consideration of program options, and the EBB expects to continue to contribute significantly to the broadened scope of the influenza program.

Clinical Trials

The expanding clinical trials programs within the NIH has stimulated development of new administrative and research technologies in study design, management of data and data monitoring and analysis. This is reflected in MIDP as well, and the EBB has been actively supporting several new initiatives, particularly two sponsored by CSB. In a multicenter study of treatment regimens for cryptococcal meningitis, EBB staff will be monitoring research data periodically for adherence to protocol and outcome. In another CSB-sponsored clinical trial, EBB staff consulted in the study design and expect to be involved with monitoring clinical data in a multicenter trial of penicillin prophylaxis of group B streptococcal sepsis in premature newborns.

EBB staff assisted the DAB bacterial vaccine program officer in two areas concerning pneumococcal vaccines. In two separate but similar trials of vaccine in prophylaxis for pneumococcal otitis media, EBB staff conducted a preliminary analysis of efficacy data and will assist in the final analysis. The larger commitment in pneumococcal vaccines, however, was Dr. Blackwelder's work with Dr. Gerald Schiffman to devise a sound but simple way to express pneumococcal polysaccharide antibody titers in the myriad of clinical trials for which Dr. Schiffman is providing the serological data. Dr. Blackwelder also assisted intramural scientists in the NIAMDD with the analysis of data from a clinical trial of pneumococcal vaccine in patients with systemic lupus erythematosis.

EBB staff provided consulation to IAIDP in two areas in FY79, including participation in the design of a study of the efficacy of skin tests to the major and minor group determinants of penicillin. The material, which was developed by Dr. Bernard Levine, can detect persons who are allergic to penicillin, but it is not known how many patients in whom penicillin is the drug of first choice are denied the drug in the basis of an unconfirmed history of allergic reaction to it. This study should help to place the history of penicillin allergy and routine use of the skin test in clinical perspective.

Ms. Jacqueline Smith continued to program for computerized analysis the Kidney Transplant Histocompatibility Study (KTHS) data. This multicenter project sponsored by IAIDP has collected a variety of clinical data judging the impact

of histocompatibility typing on the survival of kidney transplants. A preliminary analysis prepared in March showed a surprising differential in survival rates among participating centers which persisted when controlling for other factors such as the extent of tissue matching, age, and proportion of living related donors. Ms. Smith continues to be a consultant in computer programming for IAIDP staff and the contractors who are responsible for the KTHS.

Impact of Infections

In FY79 the EBB staff began with the consultant advice of Dr. Fred J. Payne a process of accessing on a routine, ongoing basis the morbidity, mortality and cost-of-illness data of allergic, immunologic and infectious diseases. This continuing project will for the first time develop these data from the same sources annually to allow a demonstration of trends and comparisons between years. Earlier results showed, as expected, the infectious diseases to be a tremendous burden to society in terms of morbidity but not mortality. This project is attempting to dissect out the the contribution of infectious disease complications to the high mortality rates of heart disease, cancer and diabetes. Previously only the underlying cause of death data were analyzed in studies of hospital records, but the extent to which infections effect adversely the course of chronic illness has not been established. A clearer understanding of the magnitude of the impact of allergic, immunologic and infectious diseases throughout the clinical spectrum expressed in terms of episodes of illness, direct cost of care, indirect costs such as days out of work or school and rates of complications in chronic illness is fundamental to the process of allocating research resources and planning research initiatives in these diseases.

Epidemiology of Nosocomial Infections

EBB negotiated partial funding of the large Study of the Efficacy of Nosocomial Infection Control Program (SENIC), a CDC sponsored study which is in the final stages of data acquisition. This comprehensive investigation of the effectiveness of standard hospital infection control procedures is expected to identify more precisely patterns of infections acquired in modern hospitals.

Trans-NIH

In the Trans-NIH areas covered by EBB staff, the Epidemiology Committee completed the draft for an NIH Epidemiology Associates Program. When implemented this program will provide three years of training in epidemiology to fifteen physicians per year. The EBB will be a preceptorship for one associate per year who will be trained in infectious disease epidemiology. Dr. Curlin represented the Institute in the NIH Diabetes Coordinating and NIH Digestive Diseases Committees.

MOLECULAR MICROBIOLOGY AND PARASITOLOGY BRANCH

The Molecular Microbiology and Parasitology Branch plans and conducts research grant, program project grant, contract, training grant, fellowship and career award programs in molecular microbiology, biochemistry, genetics, DNA recombinants, physiology, parasitology and medical entomology. It also coordinates the activities of the Parasitic Diseases Panel of the U.S.-Japan Cooperative Medical Science Program.

Appropriate Level of Support

Molecular Microbiology Section

Activity	Number	Amount
Research Grants 1/	202	$14,289,517
Research Contracts	1	337,903
Training Grants	4	322,748
Fellowships	24	285,635
Sub-total	231	$15,235,803

1/ Includes 13 Career Awards ($440,792)

Parasitology and Medical Entomology Section

Research Grants 2/	184	$11,991,405
Program Project Grant	1	349,238
Research Contracts	5	338,199
Training Grants	10	569,576
Fellowships	11	150,100
Sub-total	211	$13,398,518

2/ Includes 6 Career Awards ($197,921)

Branch	Number	Amount
Research Grants 3/	386	$26,280,922
Program Project Grant	1	349,238
Research Contracts	6	676,102
Training Grants	14	892,324
Fellowships	35	435,735
Sub-total	442	$28,634,321

3/ Includes 19 Career Awards ($638,713)

Molecular Microbiology

Subsequent to the submission of the FY 1978 Annual Report, Dr. Delappe was a student in a course at Aspen, Colorado where he learned the Maxam-Gilbert method of DNA sequencing, including isolation of restriction fragments, labeling with polynucleotide kinase, specific chemical cleavages, and gel display of the sequence.

During the year a number of scientific meetings, both foreign and domestic, relevant to the research supported by this Section were attended. A meeting on Recombinant DNA and the Eukaryotic Genome, sponsored by the French National Institute of Health and Medical Research (INSERM), was held in the village of Blois, France in November, 1978. A great many aspects of recombinant DNA research and their impact on eukaryotic genome structure and function were discussed in the presence of an international audience. The meeting was a productive and profitable one to attend.

In June, 1979, Dr. Delappe gave an opening lecture at the Fourth International Symposium on Antibiotic Resistance at the Castle Smolenice near the city of Bratislava, Czechoslovakia. The proceedings will be co-published by AVICENUM (Czechoslovak Medical Press, Prague) and Springer-Verlag, (Heidelberg, Germany).

In May, Dr. Delappe was asked to become a member of the Project Advisory Group of the Bureau of Drugs, FDA. This is the immediate advisory group for BOD research contracts. He was also asked to become a member of an FDA study section, but elected not to accept the appointment.

In October, an announcement was made of the forthcoming award of a Nobel Prize to Dr. Hamilton Smith of The Johns Hopkins University. Dr. Smith shared the prize with two other scientists. The award recognized the development of restriction endonucleases, enzymes that can be used to study genetic organization and to manipulate DNA. This was one of the origins of the recombinant DNA technology. At the time he made his contribution (in 1969), Dr. Smith was supported by a Research Career Development Award and a research grant, both of which were funded by the National Institute of Allergy and Infectious

Diseases. He was the tenth grantee of the branch to receive the Nobel Prize.

Program Summary

In addition to the basic free-ranging research of relevance to the Institute there are two structured programs supported by this section of the Branch, one of which involves mechanisms of resistance to antimicrobial agents and the other, recombinant DNA.

(1) Mechanisms of Resistance to Antimicrobial Agents

During the past ten years, the problem of microbial resistance to therapeutic agents has become increasingly apparent. The relevance of this phenomenon to other interests of this Institute such as hospital-associated infections due to staphylococci, gram negative bacteria, and other organisms, together with investigations of gonococci and pneumococci, is obvious.

At this point, there is substantial clinical and epidemiological evidence that development of antibiotic resistance, and especially plasmid (extrachromosomal) mediated drug resistance, represents a major problem in medical care. Most of the projects are concerned with defining the molecular and biochemical basis for resistance, the principal goal being to elucidate the fundamental biological mechanisms involved in the development of drug resistance by microorganisms. Specific goals involve investigation of the origin, development, evolution, expression and mechanism of drug resistance in a variety of specific microorganisms. Examples of microorganisms of particular interest are (but not limited to): Enterobacteriaceae, Pseudomonas, Neisseria, staphylococci, streptococci, mycobacteria, mycoplasmas, and pathogenic fungi.

Research Highlights

AI 14938-02 C. Cech (University of Colorado): This investigator has studied the mechanism of rifampicin inhibition of Escherichia coli RNA polymerase with a newly developed steady state assay for RNA chain initiation and by analysis of the products formed with several 5'-terminal nucleotides. The major effect of rifampicin was found to be a total block of the translocation step that would ordinarily follow formation of the first phosphodiester bond. These effects were incorporated into a steric model for rifampicin inhibition. Additional minor effects of the enzyme bound inhibitor were to increase slightly the lifetime of RNA polymerase on the λP_R promoter and to increase by two the apparent Michaelis constants of the initiating triphosphates. The products formed by RNA polymerase in the presence of rifampicin belong nearly exclusively to the class triphosphate purine phosphodiester nucleotide. No evidence for the accumulation of such molecules was obtained in vivo.

AI 10971-08 M. Higgins (Temple University School of Medicine): Dr. Higgins has examined the morphological effect of the antibiotic cerulenin on cell shape of Streptococcus faecalis. This drug, which inhibits fatty acid synthesis, causes an elongation of growth zones and a blockage of cell division similar to that observed when DNA synthesis is inhibited in these cells. This is an interesting observation in that he had predicted that a signal at the

end of a round of DNA synthesis is necessary for an inhibition of autolytic activity and a concomitant stimulation of cell division. Recently, it has been shown that a lipid intermediate is involved in a reduction of autolytic activity is this organism, and it is supposedly at this level that cerulenin works to block cell division.

AI 12835-05 R. Martinez (University of California, Los Angeles): Studies involving the bactericidal capabilities of normal rabbit serum (NRS) have revealed the presence of two principal components capable of reducing the viability of Bacillus subtilis, in vitro. These components, rabbit lysozyme and a non-lysozyme factor, comprise the classical β-lysin system in rabbits, long noted for its heat-stability and principal activity against gram positive bacteria. Both of these factors have been purified to homogeniety, examined for bactericidal activity (singly, and in combination), and have been (or are in the process of being) characterized at the molecular level. The results indicate that the primary bactericidal component of NRS active against a variety of organisms is a single, low molecular weight, proteinaceous factor, presumably released from platelets during the coagulation process. Although the secondary factor, lysozyme, has also been shown to exhibit bactericidal activity, it has been demonstrated that when examined at concentrations found in dilute NRS, the presence of this enzyme can account for only a minor portion of the antibacterial action observed for NRS. These results have been confirmed in whole serum studies to which specific inhibitors of lysozyme enzymatic and bactericidal activity have been added.

AI 12500-05 J. Murphy (Harvard Medical School): During the past year he has published a series of experiments that have demonstrated for the first time that iron has a direct effect on inhibition of diphtheria toxin production by cells that were "derepressed" for toxin. In addition, it has been demonstrated that the decay kinetics of toxin release were identical following either iron or rifampicin. By preventing reinitiation of RNA polymerase, the decay kinetics imposed by rifampicin block mimic transcriptional control. Thus, the similarity of inhibition kinetics of toxin release imposed by iron and rifampicin suggested that the control of toxin production was at the transcriptional level. Further evidence to support this hypothesis was provided by RNA:DNA hybridization experiments. He has shown that there is a marked stimulation of hybridization of total ^{3}H-RNA extracted from iron starved corynebacteria to β-phage DNA. Total labeled RNA extracted from cells that were in iron excess or from the non-lysogenic, non-toxigenic C. diphtheriae C7 (-) hybridized to the same extent.

AI 11756-12 C. Gilvarg (Princeton University): This investigator has isolated the major component of the cell wall of Bacillus megaterium M46, and his studies indicate that it is a complex polysaccharide over twenty times the size of previously reported teichuronic acids. The molecular size and shape of this unusual teichuronic acid were elucidated by a combination of hydrodynamics, chemical and electron microscopic studies. This should contribute to a greater understanding of the synthesis and assembly of the bacterial cell wall which plays an important role in resistance to antimicrobial agents.

AI 10311-10 D. Dubnau (Public Health Research Institute of the City of New York): This investigator has reported the construction of several plasmid chimeras by molecular cloning, thus demonstrating the utility of the B. subtilis system for recombinant DNA experiments and providing a collection of new plasmids for studies on replication, incompatibility, and transformation, as well as for the engineering of better cloning vectors. A plasmid pE194, obtained from Staphylococcus aureus confers resistance to macrolide, lincosamide, and streptogramin type B ("MLS") antibiotics. For full expression, the resistance phenotype requires a period of induction by subinhibitory concentrations of erythromycin. A copy number in the range of 10 to 25 copies per cell is maintained during cultivation at 32°C. It is possible to transfer pE194 to Bacillus subtilis by transformation. In B. subtilis, the plasmid is maintained at a copy number of approximately 10 per cell at 37°C, and resistance is inducible. Tylosin, a macrolide antibiotic which resembles erythromycin structurally and to which erythromycin induces resistance, lacks inducing activity. Two types of plasmid mutants were obtained and characterized after selection on medium containing 10 μg of tylosin per ml. One mutant class appeared to express resistance constitutively and maintained a copy number indistinguishable from that of the parent plasmid. The other mutant type had a 5- to 10-fold-elevated plasmid copy number (i.e., 50 to 100 copies per cell) and expressed resistance inducibly. Both classes of tylosin-resistant mutants were shown to be the result of alterations in the plasmid and not to modifications of the host genome.

(2) Recombinant DNA Molecular Research

During the past few years, the development of certain techniques in the area of molecular biology has made it possible to construct functional DNA molecules in vitro which contain segments derived from diverse biological sources. The scientific innovations which led to this technological breakthrough were mostly derivative from basic studies on the mechanism of restriction, which normally acts as a barrier to gene flow among microorganisms, and on the molecular biology and genetics of bacterial plasmids, especially those specifying drug resistance. Grantees of this Institute played a preponderant role in both of these areas. Whereas DNA recombination in nature has depended on random processes, the experimental techniques now available enable the in vitro construction and subsequent replication of DNA molecules needed for specific experimental goals. This basic advance, coupled with refinements of the existing technology, should provide greater knowledge of the mechanisms of pathogenicity (at the molecular level) of viral, bacterial, mycotic and parasitic agents. This information, in turn, may lead to improved prevention, diagnosis and treatment of infectious diseases. Notwithstanding the potential benefits of recombinant DNA molecule research, there may be associated potential biohazards which must be avoided by the design, construction and testing of safer host-vector systems for use in these studies.

The primary goal of this research is the development and utilization of recombinant DNA molecule technology to increase fundamental knowledge and ultimately enhance control of the etiological agents of infectious diseases. Another goal is the synthesis of a variety of biologically useful substances through the construction of bacterial cells containing functional DNA of either plant

or animal origin. An equally important goal is the identification, assessment and elimination of any and all potential biohazards encountered in the exploitation of this technology.

Research Highlights

AI 10311-10 D. Dubnau (Public Health Research Institute of New York): The ability to carry out molecular cloning in Bacillus subtilis would be useful for a variety of studies on sporulation, transformation, and gene expression. In addition, such a capability might be industrially significant, because Bacillus species are of considerable commercial importance. Antibiotic resistance chimeric plasmids have been constructed by in vitro enzymatic manipulation and introduced into Bacillus subtilis by transformation. The parental plasmids used had been introduced into B. subtilis from Staphylococcus aureus by transformation. Seven recombinant plasmids have been constructed using restriction endonucleases. Although all of the recombinant plasmids replicate and express their antibiotic resistance characters, three of them have suffered a loss of DNA, either in vitro or, more likely, in vivo. The deletion event in all cases involved one of the two termini used to join the parental plasmids. The plasmid chimeras reported should prove useful for the study of plasmid replication, incompatibility, and recombination. In addition, the utility of the B. subtilis system for molecular cloning has been clearly illustrated.

AI 8619-12 S. Cohen (Stanford University Medical School): DNA fragments generated by the EcoRI or HindIII endonucleases from the low copy number antibiotic resistance plasmids R6 and R6-5 were separately cloned using the high copy number ColE1 or pML21 plasmid vectors. The hybrid plasmids that were obtained were used to determine the location of the EcoRI and HindIII cleavage sites on the parent plasmid genomes by means of electron microscope heteroduplex analysis and agarose gel electrophoresis. Ultracentrifugation of the cloned fragments in cesium chloride gradients localized the high buoyant density regions of R6-5 to fragments that carry the genes for resistance to streptomycin-spectinomycin, sulfonamide, and mercury and a low buoyant density region to fragments that carry the tetracycline resistance determinant. Functional analysis of hybrid plasmids localized a number of plasmid properties such as resistance to antibiotics and mercury and several replication functions to specific regions of the R6-5 genome. Precise localization of the genes for resistance to chloramphenicol, kanamycin, fusidic acid and tetracycline was possible due to the presence of identified restriction endonuclease cleavage sites within these determinants.

AI 8619-12 S. Cohen (Stanford University Medical School): Messenger RNA that encodes the common peptide precursor for the hormones corticotropin and β-lipotropin was purified from the neurointermediate lobe of bovine pituitaries, and double-stranded complementary DNA species synthesized from this template were cloned in Escherichia coli χ1776 by inserting them into the Pst I endonuclease cleavage site of the pBR322 plasmid. Certain of the cloned cDNA inserts contain nucleotides corresponding to the complete amino acid sequence of bovine corticotropin and a coding sequence that corresponds to at least the first portion of bovine β-lipotropin. The nucleotide sequences

coding for corticotropin and β-lipotropin are separated on the cDNA by a 6-base-pair sequence encoding lysine and arginine, indicating that the carboxyl terminus of corticotropin is connected on the precursor peptide with the amino terminus of β-lipotropin by these two amino acids. In addition, the cloned cDNA insert is characterized by an usually high C+G (cytosine and guanine) nucleotide base content as well as by a number of DNA sequence duplications.

KO4 AI 119-03 P. Lovett (University of Maryland): The technique of molecular cloning involves the in vitro insertion of fragments of DNA into small replicons, plasmids, or phage genomes, followed by selection of chosen recombinant molecules by transformation of appropriate recipient cells. Direct application of recombinant DNA technology to the study of Bacillus subtilis will ultimately provide a general method for constructing partial diploid strains which will, in turn, permit genetic complementation analyses of specific mutations and provide a source of easily obtainable DNA highly enriched for genes of chromosomal origin whose in vitro expression may be of special interest such as sporulation genes. Plasmid pUB110, originally detected in Staphylococcus aureus, specifies resistance to neomycin and has been transformed into Bacillus subtilis 168. In B. subtilis, pUB110 is stably maintained at about 50 copies per chromosome and renders the host resistant to neomycin sulfate at 5 μg/ml. pUB110 was transferred by transduction from B. subtilis to strains of B. pumilus and B. licheniformis.

Parasitology

On September 18-20, 1978 the Branch was represented at a "Symposium on Waterborne Transmission of Giardiasis" sponsored by the U.S. Environmental Protection Agency. The goal of the symposium was to discuss the state of the art regarding Giardia species and the disease as they relate to water supplies, and to identify areas where further research is needed. The topics discussed included the organism, the disease, epidemiology, detection methodology and water treatment technology. It was concluded that more research on all aspects of giardiasis is needed.

The 13th Joint Conference of the U.S.-Japan Parasitic Diseases Panel was held in Okayama, Japan on October 4-6, 1978. Forty-seven Japanese and ten American scientists attended this conference at which 42 papers were presented on a broad range of studies of both schistosomiasis and filariaris. In addition to the formal scientific sessions, many of the U.S. participants took advantage of opportunities to develop potential collaborative research relationships with their Japanese counterparts.

The Branch was also represented at a conference on "Pharmaceuticals for Developing Countries" sponsored by the National Academy of Sciences on January 29-31, 1979. Particularly relevant to the Parasitology program were the presentations on cellular regulatory processes in parasitic helminths and those on the pharmacological exploitation of biochemical differences between parasites and hosts.

Late in 1978, a Request for Application for Tropical Disease Research Units program project grants was issued. The basic objective of this new initiative is to bring together relevant biomedical knowledge and technology in a multi-disciplinary attack on the world's tropical and parasitic diseases. Nine applications were received and reviewed in June, 1979 and, following the October, 1979 Council, it is hoped that funds will be available to permit the funding of 2 or 3 of these program projects.

On June 15, 1979, an RFA was issued for New Investigator Research Awards in Tropical Diseases. To help bridge the transition from training status to that of a productive investigator, this special grant program will provide support for young scientists and physicians with meritorious ideas for research on the world's tropical diseases. Emphasis will be placed on research on malaria, schistosomiasis, filariasis, trypanosomiasis, leprosy, leishmaniasis and the immunology of these diseases. The first of these awards will be made in July, 1980.

Program Summary

In addition to the basic free-ranging research of relevance to the Institute, there are two structured programs supported by this section of the Branch--biological regulation of vectors and immunology of parasitic infections.

(1) Biological Regulation of Vectors

This program has as its goal the advancement of fundamental studies which might lead to effective methods of biological regulation of vectors. For the past 30 years, control of pests and disease vectors has been based primarily on the use of synthetic organic compounds which had the "advantages" of long residual action and toxicity to a broad spectrum of target organisms. It has now been shown that because of these very characteristics, many of these pesticides are more deleterious than beneficial, when all effects on man and his environment are considered. Furthermore, resistance to broad spectrum chemical pesticides has reduced their effectiveness in many vector control programs. For these reasons the search for alternative methods of pest control has become imperative, and it is generally agreed that the best approaches will consist of integrated pest management programs which combine biological control, in the broadest sense, with the judicious use of more specific chemicals and management of the physical environment. This approach to vector control must be based on adequate information about the ecology of the target organism, the environment in which the control program is to be conducted, effects of control measures on non-target organisms in the environment, and the biology of the disease organisms being transmitted.

Research Highlights

AI 10986-06 D. Sonenshine (Old Dominion University): The identity of the female sex pheromone (2,6-dichlorophenol) of the ticks Dermacentor andersoni and D. variabilis was confirmed. Dr. Sonenshine also demonstrated the role of foveal glands as the source of this compound. Pheromone-impregnated materials, especially dusts, caused delay of mating and confusion of mate-

seeking males. The use of sex pheromones in an integrated control program would provide a negative pressure effect on the reproductive capacity of the tick population surviving the direct effects of insecticides.

AI 11847-11 R. Barr (University of California, Los Angeles): Dr. Barr has found that Wolbachia pipientis, a rickettsia-like symbiont of Culex pipiens, affects the sperm of male mosquitoes in such a way that the sperm are not able to fertilize eggs unless the eggs are also infected with Wolbachia. If the eggs are infected with Wolbachia of different geographic origin, the cross may be incompatible. Research is continuing to determine whether Wolbachia might be cultured and used as a means of controlling mosquito reproduction.

AI 11123-07 N. Alger (University of Illinois): Dr. Alger has been studying the use of the microsporidian Nosema algerae as a biocontrol agent against Anopheles mosquitoes. Her most recent studies in Pakistan have shown that it is possible to infect field populations of mosquito larvae, and that the resultant adult mosquitoes will produce fewer eggs, some of which will be infected and will not live long enough to transmit malaria. Non-target aquatic invertebrates are not infected; thus, the use of N. algerae in mosquito biocontrol appears to be safe.

AI 13295-03 C. Bayne (Oregon State University): In a study of the susceptibility of the vector snail Biomphalaria glabrata to Schistosoma mansoni, Dr. Bayne has discovered that sporocyst-killing cells isolated from the snails are actually amebae of the genus Nuclearia which attach to the surface and penetrate the schistosome sporocysts. These amebae-sporocyst interactions may be important determinants of snail susceptibility in nature.

(2) Immunology of Parasitic Infections

The complexity of structure and function of parasites has made the study of the immunology of these infectious agents exceptionally challenging and rewarding. Exciting opportunities for the elucidation of mechanisms and manifestations of immunological responses to parasites now exist as the result of the impressive developments in immunology in recent years. Major ultimate goals of studies on the immunology of parasitic infections are the development of effective vaccines for the prevention of parasitic diseases (such as malaria, schistosomiasis and filariasis), the intervention in the host response to prevent or ameliorate disease processes which are immunologically mediated, and the development or improvement of immunodiagnostic procedures for parasitic infections, especially as they relate to the immune status of the host.

A related goal of these studies is to contribute to an understanding and solution of basic and clinical problems associated with other disease entities, especially immunological disorders and hypersensitivity states. A number of parasitic infections are excellent models for such studies as: (a) the mechanisms of intracellular immunity, (b) the enhancement or suppression of concurrent infections or tumor development, (c) immunopathological mechanisms, (d) development of disease processes in immunosuppressed or immunostimulated hosts, (e) the biochemical and genetic mechanisms for the development of

pathogen variants with different immunological characteristics, (f) the genetic basis for variations in host response and (g) the role of IgE and other cytotropic antibodies in hypersensitivity.

Research Highlights

AI 15235-02 H. Shear (New York University): In a study of macrophage activation in experimental malaria (P. berghei), Dr. Shear showed that early in an infection macrophages appear to be activated and parasitized erythyocytes are ingested; later, the macrophages appear to be "blocked." Indirect evidence points to the possibility of inhibitory immune complexes in the serum of infected animals. An analysis of this blocking activity should help clarify the animals' inability to cope with the disease ultimately.

AI 12770-04 M. Wittner (Albert Einstein College of Medicine): In a study of mechanisms of immune resistance to Trypanosoma cruzi, Dr. Wittner has investigated the role of macrophages in the in vivo resistance to T. cruzi infection. Mice genetically resistant to T. cruzi were depleted of peritoneal macrophages prior to infection with the parasite. Increased parasitemia and mortality in this group are consistent with the hypothesis that the macrophage constitutes an early line of defense in T. cruzi infection.

AI 12663-05 J. Farrell (University of Pennsylvania): Using the golden hamster as a model for the study of Kala-azar, Dr. Farrell has shown that hamsters inoculated intracardially with L. donovani develop fatal visceral infections whereas animals injected intradermally usually exhibit self-limiting, non-ulcerating cutaneous infections and subsequent resistance to reinfection. He is currently investigating the immunological parameters of these different manifestations of the disease.

AI 08989-09 W. Trager (Rockefeller University): In a series of preliminary experiments, Dr. Trager and his colleagues have developed in vitro techniques for the collection of relatively pure merozoites of Plasmodium falciparum to be used in antigen-antibody studies. Aotus monkeys immunized with this in vitro culture-derived antigenic material are protected from challenge infection.

AI 12913-03 D. Boros (Wayne State University): Dr. Boros has shown that the granulomatous inflammatory response around schistosome eggs is mediated by inflammatory T lymphocytes. During the chronic phase of the infection certain lymphocytes assume a suppressive/regulatory role and exert their influence on the inflammatory lymphocytes to diminish their activity which results in smaller granulomas and hence diminished host pathology.

(3) General Parasitology Research Highlights

AI 14294-03 J. Bennett (Michigan State University): In a study of the effects of chemotherapeutic agents on schistosome physiology, Dr. Bennett has demonstrated that the effective schistosomacide, praziquantel, causes a massive contraction of the worm and a large influx of calcium. Apparently the drug acts by stimulating the influx of calcium which causes the muscle contractions.

AI 15919-01 J. Dubey (Montana State University): In an investigation of a human toxoplasmosis outbreak in a stable, Dr. Dubey found that of the 37 cases reported, none had had common meals; thus raw meat could not be implicated in the transmission. Apparently the infections occurred either via the hand-to-mouth or dust inhalation route, and the source of infection was a group of Toxoplasma positive cats which lived in the stable area.

AI 10250-08 L. Ash (University of California, Los Angeles): Further progress has been made in the development of a primate host for the human filarial parasite, Wuchereria bancrofti. In addition to establishing patent infections in nine monkeys, one of these infections represented the first serial passage of the parasite from monkey to monkey.

AI 14718-02 R. Cypess (Cornell University): There has long been a need for a specific and reliable diagnostic test for toxocaral visceral larval migrans (VLM) since this syndrome presumably may be caused by a variety of helminths. Dr. Cypess has demonstrated that the enzyme-linked immunosorbent assay (ELISA) is superior to indirect hemagglutination, bentonite flocculation and double diffusion in agar tests. Based on specificity, sensitivity and positive and negative predictive values, the ELISA appears to be the serodiagnostic method of choice for toxocaral VLM.

AI 11942-05 M. Müller (Rockefeller University): Dr. Müller, in a study of the biochemical cytology of Trichomonas vaginalis, has shown that drug-resistant strains of the parasite do not take up metronidazole in the presence of oxygen. These studies suggest that the mechanism of resistance is connected with alteration in the effect of oxygen on the intracellular reduction-oxidation system.

Contract Activity

The schistosomiasis supply contract service, located at the University of Lowell, has continued to be utilized extensively by most schistosomiasis researchers in this country. This invaluable service can provide all the human schistosome parasites and their vector snails.

The filariasis supply contract, located at the University of Georgia, has continued to provide filariasis research workers with various filarial parasites and their vectors. The complexity of filarial life cycles and the special facilities needed for the housing and rearing of arthropod vectors makes this service contract especially valuable to researchers who otherwise might not be able to pursue their research interests. Acceleration of research in filariasis is in part directly attributable to this service.

Two research contracts involving the development of techniques for the cryopreservation of various stages of human and animal filariae are currently in progress. Short- and long-term cryopreservation of microfilariae and infective-stage larvae of both sheathed and unsheathed microfilarial species has been accomplished, and this now provides unique opportunities to transport rare and scarce materials to laboratories capable of working with various aspects

of these parasites. In particular, work on Wuchereria bancrofti and Brugia malayi infections has been enhanced considerably.

As a result of a recommendation made at an NIAID workshop on the "Radiobiology of Schistosomes and Filariae" held at NIH on April 17-18, 1978, an RFP was issued for a contract for the development of techniques for the radio-labelling of schistosome and filarial larvae. Six proposals were received and reviewed, and a contract was awarded to Cornell University on June 29, 1979 for two years.

EXTRAMURAL ACTIVITIES PROGRAM

NIAID

TABLE OF CONTENTS

REPORT OF THE DIRECTOR, EAP

A. Introduction

The Extramural Activities Program (EAP) of the NIAID ended the decade of the 70's by awarding the largest number of regular research projects of any year during the decade, although the number of trainees supported was smaller during the early 1970's. The EAP workload, associated with the increased number of applications and proposals received, was increased by about 25%. This overload was handled by part-time employees, overtime, and the increased productivity of all the employees. Although there were stressful times when it seemed we would not meet our assigned schedules, it was an exciting time to be in the EAP.

B. The Review of Research Proposals

The Program and Project Review Branch was fully staffed for the first time since the reorganization of the Institute. To assist in the tremendous workloads of the winter and spring rounds, reinforcements were received from the Office of the Director, EAP and from the Office of the Director, NIAID. This workload is described later in the report from that Branch. All of these reviews have resulted in funding of those applications and contract proposals which were approved with good priority scores. The staff in this Branch has been congratulated for their efforts.

C. Implementation of the Multi-Axis Coding System (MACS)

In Fiscal Year 1979, we introduced and began accumulating data by the new multi-axis coding system. This new and more complex system of record keeping was brought about at the recommendations of the Data System Planning Committee to meet Institute requirements for broader scientific data and to meet NIAID, NIH and other requirements for administrative data. This activity also required considerable extra effort on the part of staff because it was a new system which was implemented during the year in which NIAID received and reviewed the largest number of application and contract proposals to date.

This system recommended by the Data Systems Planning Committee, as reported in last year's annual report now includes four axes of biological data; administrative subjects such as basic, applied and development (BAD); scientific base, applied technology transfer, and training (SATT); and coding for human subjects, clinical trials and prevention. This is a management data system and will eventually be evaluated on that basis.

D. Personnel

There were fourteen departures from the EAP and fourteen new appointments during FY 1979. Some of these new appointments were temporary appointments which terminated in 1979. The lag time for the process of

appointing new employees remains about the same as it was last year, meaning that there were approximately forty man months during which there were employee vacancies in the EAP. This represents over three man years and considerably hampers the accomplishment of EAP objectives.

Retirements

Mildred Applestein
Gwen Northcutt
Eleanor Wyatt

Employees who have left EAP

Virginia Alary
Mary Baldwin
Kathy Cherry
Sue Connors
Maria Decker
Melvin Joppy
Cynthia Jose
Carol Kimmel
Harry Levine
Janice Pusey
Earle Vance

New Appointments

Todd Ball
Betty Bucher
Bonnie Dunning
Diane Edwards
Melo Ellis (Stay-in-School)
Debbie Frazier (Temp)
Carla Goldblum
John Hamill
Fran Hisaoka
Annette Hobbs (Expert Consultant)
Anne Rabon
Sara Spencer
Gary Thompson
Daryl Thornton (Stay-in-School)
Delores Walton (Temp)

Awards (Quality Increase)

Louis Bourgeois
Gertrude Cohen
Grace Ellis
Fran Schlademan
Hubert Sumner
Rick Wiener

Training

Fifty-five training courses at a cost of just over $5,000 were taken by staff.

Figure I shows the Organizational Chart.

E. Manpower Development

The traditional training program increased insofar as postdoctoral awards were concerned. The trend in predoctoral awards continued at a slight decline. There was a continued decline in the number of physicians who were interested in research training and this loss of potential clinical investigators remains the major problem in the NIAID manpower development program as well as in other similar programs at the NIH.

The amendment to the National Research Service Act (NRSA) which shifts more support to institutional grants and less to individual fellowships has not made a significant impact this fiscal year. There are 81 T32 training grants now being administered by NIAID. Of these, 34 are in the IAID program and 47 in the MID program.

Table I estimates the total number of trainees being supported by NIAID on the authority of NRSA and on the old T01 programs of which there are 18 still operating on unexpended funds and support 70 fellows.

TABLE I

All Trainees - Estimated

	PROGRAM		
	IAIDP	MIDP	TOTAL
Predoctoral	40	65	105
Postdoctoral	119	202	321
M.D.	57	48	105
TOTAL	216	315	531*

*Includes T01

Table II estimates the number of individual fellows being supported in each of the major programs. The number of M.D.'s in comparison to the number of Ph.D.'s is also shown.

TABLE II

Individual Fellowships (F32)

	PROGRAM		
Degree	IAIDP	MIDP	TOTAL
Ph.D.	55	94	149
M.D.	9	8	17
TOTAL	64	102	166

Table III estimates the number of trainees both predoctoral and postdoctoral in the IAID and MID programs.

TABLE III

Institutional Training (T32)

	PROGRAM		
	IAIDP	MIDP	TOTAL
Predoctoral	37	68	105
Postdoctoral	97	145	262
TOTAL	134	213	367

F. Decline of Physicians in Research Training

The decline in M.D.'s selecting research as a career still remains of concern to those involved with biomedical training. One aspect which appears not to have been examined is the long-range effect of legislation. Legislation focusing on the production of more physicians for medical service began in 1963. It evolved into a federal initiative that became increasingly focused on training. The history of this legislation was as follows:

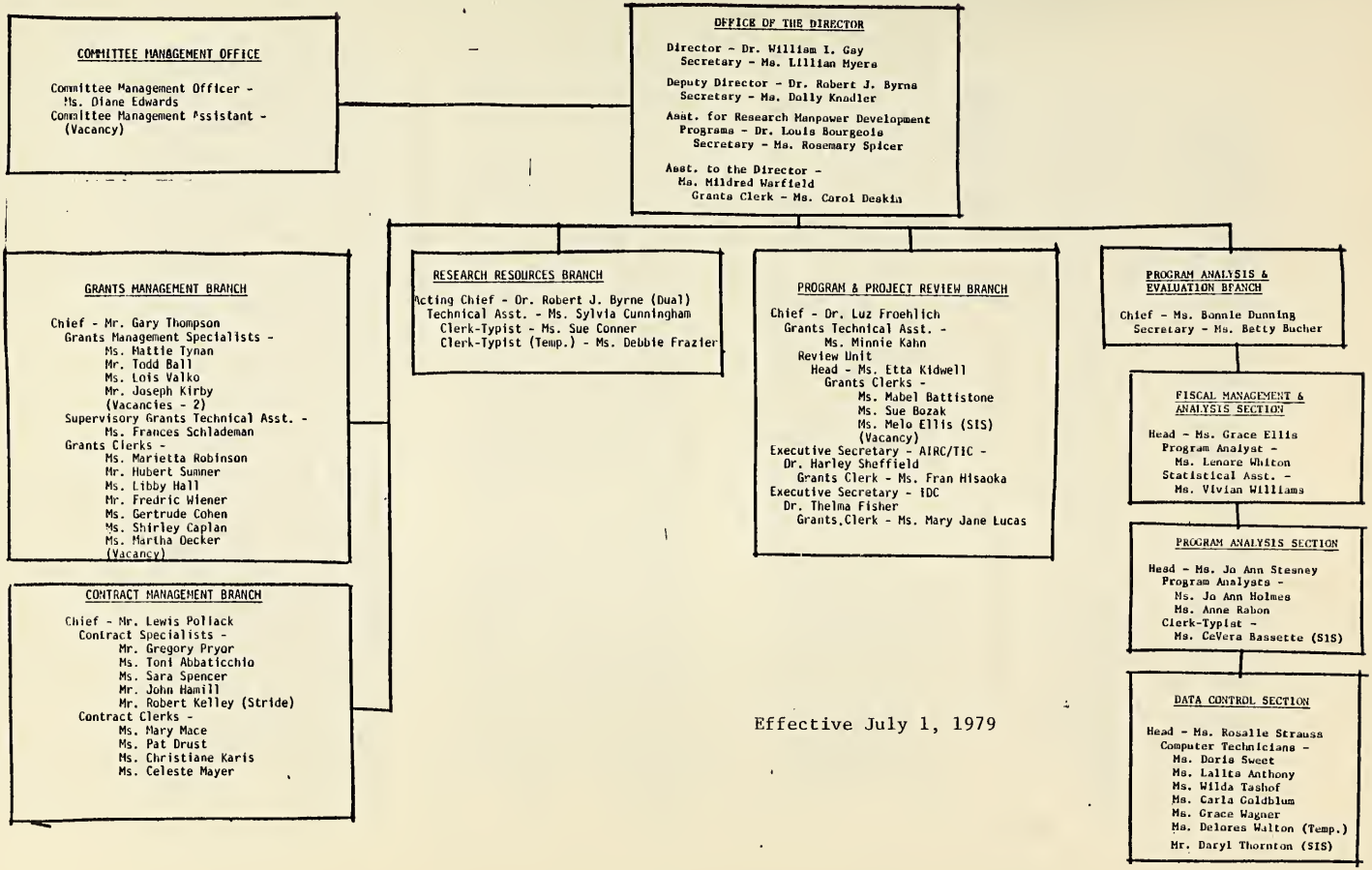
1963, Health Professional Educational Assistance Act provided for construction grants and loans for undergraduate students,

1965, amendments to the Act provided aid for scholarship and medical schools operating costs,

1968, the Health Manpower Act emphasized absolute increase in medical school enrollments,

1971, Comprehensive Health Manpower Training Act designed to increase enrollment through capitation, and to increase practitioners in rural and shortage areas and family medicine,

ORGANIZATIONAL CHART FOR OPERATING PURPOSES



Effective July 1, 1979

1976, Health Professions Educational Assistance Act, P.L. 94-484, established a target of 50% of graduating M.D.'s as primary care specialists, and to encourage medical care institutions to establish residencies in family practice, and primary care specialties.

These federal actions, plus the leveling of research budgets, the low level of stipends, and a pay back requirement for research training have made research much less attractive than service for the graduating M.D. In the 1980's, the NIH must reverse this trend.

G. Manpower Survey being Conducted by the American Society for Microbiology

To date, the Society has received about 17,000 responses to the 25,000 questionnaires sent out. This was 7,000 more than was anticipated by the designers of the survey. These have been coded and they are being keypunched and computerized. Dr. William Epstein, the technical writer, and Mr. John Dirkse the statistician, are working to examine the data and prepare a draft of the report. The work on the survey is ahead of schedule. A recent supplement has been awarded to assist in anslysis of the data acquired from the additional responses received.

H. Committee Management

In Fiscal Year 1979, the Committee Management Officer retired and her assistant left for another position. The Committee Management Officer's position was filled just prior to the May Council by a very capable employee and the position of Assistant to Committee Management Officer could not be filled in the fourth quarter because of a freeze on personnel. This meant that this office also had to operate with a series of temporary measures for accomplishing typing, filing, review of vouchers and travel claims and travel activities. It is a tribute to the new committee management -- council secretary that no major problems resulted from operating an office as important as that of committee management and council services in this manner.

I. Conference Proposal Review

The EAP review of all "unsolicited" conference proposals, i.e., those arising outside the program areas of NIAID, which was successfully initiated and implemented in FY '78, has continued at about the same level in FY '79. Prospecti were received from the Fogarty International Center, as well as directly from the proposers. Those which were accepted for review, primarily on the basis of Institute relevance, were subjected to a primary review by an ad hoc scientific group and a secondary review by the Research Contract Review Group, the latter being scheduled once each quarter. A maximum amount of funds was set aside for the support of these conferences. Eight R-13 conference grant applications were also received and reviewed in FY '79.

They received a primary No Study Section (NSS) assignment but were peer reviewed by the EAP; these were presented at the appropriate Council meetings for final review and approval. A total of 69 conference prospecti and R-13 grant applications were received by EAP in FY '79. Of this number, 39 were reviewed and 35 of these were approved for funding and/or sponsorship for a maximum of $276,796. Eleven of the 69 will be reviewed for possible FY '80 funding. (See Table IV).

TABLE IV

CONFERENCE/WORKSHOP PROSPECTI REVIEWED BY NIAID
FY '79

Received.....69

From Fogarty International Center (FIC).....59

Sponsorship & Funds.....58
Sponsorship Only..... 1

Directly by Institute.....2

From DRG (R13's).....8

Not Reviewed.....19

Returned to FIC due to lack of Institute relevance..15
Returned to FIC due to Institute policy..... 3
Returned to FIC due to lack of justification for meeting site..... 1

Reviewed.....39

Approved.....35
Sponsorship & Funds.....30*
Sponsorship Only..... 1
Approved (No Funds)..... 4

Disapproved (NIAID and/or FIC).....4
Sponsorship & Funds.....4
Sponsorship Only.....0

To Be Reviewed.....11

FUNDED OR SCHEDULED FOR FY '79 FUNDING (Thru May '79) **

	IAIDP		MIDP		Other NIAID		TOTAL	
	#	$	#	$	#	$	#	$
R-13 Grant Applications.....	1	$18,125	6	$80,983	0	0	7	$ 99,108
Other Extramural Prospecti.....	8	$75,050	9	$76,638	5	$26,000	22	$177,688
TOTAL.....	9	$93,175	15	$157,621	5	$26,000	29	$276,796

*Includes 8 R-13's some of which will not be paid
**Includes some which were reviewed in FY '78 but funded in FY '79

I. CONTRACT MANAGEMENT BRANCH

The Contract Management Branch provides management services to the Institute's Research Program including solicitation, negotiation, award, and administration of all Institute research contracts. The CMB will continue to implement contract policies and procedures promulgated by higher procurement authority.

During FY 1979, Mr. Harry LeVine, Contract Specialist, resigned in order to further his education. His replacement was Ms. Sara Spencer formally of NINDS. Mrs. Mildred Applestein, Contracting Officer, retired after 30 years of Government service. Her replacement was Mr. John Hamill formally of NCI.

In accordance with Secretary Califano's initiative to correct major deficiencies in the contracting process, CMB is required to prepare the following reports:

1. Justification for Non-Competitive Procurement (JNCP) approved monthly.

2. Non-competitive Procurement and Scheduling of R&D contracts - monthly.

3. Degree of competition (quarterly).

4. Even distribution of contract awards within the fiscal year plus variances (quarterly).

5. Major contracts over $500,000 (semi-annual).

6. Close-out of contracts (quarterly).

One of the principal objectives CMB achieved during FY '79, was to spread the workload more evenly through the fiscal year by shifting contract renewal dates and new contracts as equally as possible in each quarter.

It is estimated that there will be 190 active contracts with a FY '79 allocation of $16,000,000.

II. GRANTS MANAGEMENT BRANCH

Fiscal Year 1979 was a year of considerable hardship for the GMB as the Branch lost the equivalent of one full-time professional and one half-time administrative/award clerk, resulting from vacancies left unfilled for appreciable periods of time. Nonetheless the Branch was responsible for approximately 1,700 awards with a dollar value in excess of $115,000,000 during this reporting period. The GMB is responsible for the fiscal and administrative management of all grants and awards issued by the NIAID. The GMB works very closely with the programmatic divisions and provides the fiscal and administrative expertise necessary to more effective program management. The GMB also serves as an interpreter of grant policy and procedure issued by the several echelons within the DHEW.

There were many policy and procedural changes that impacted significantly on the operations of the Branch. Perhaps two of the more significant changes were, 1) the loss of the categorical Report of Expenditures, which will be replaced by the non-categorical Financial Status Report; and 2) in a more favorable change, the initiation of the new project-period concept which, simply stated, enables the awarding component to carryover unexpended direct cost funds from one project-period to another, and/or, to use the total unexpended direct cost balance from one project-period as a funding offset to another.

The staff of the GMB has made significant contributions as members and chairpersons of policy and procedure work groups, committees and subcommittees both at the NIAID and at the NIH. Examples of such activity include: NIH grants management self-appraisal, NIH change of institution policy, PHS grants policy statement, refinement of procedures for development of recommended grant budgets on those grants assigned to PPRB (NIAID) for competitive review, and various other grant policy and procedure drafting committees. Additionally, Branch personnel have participated as faculty members or attendees at seminars where grantee personnel were in attendance, such as the NIH-AAMC Workshop on Business Affairs, the AAMC-group on Business Affairs-Continuing Education Program, and the several Grants Management Workshops conducted by the GMAC for grantee institutions and NIH personnel alike.

Generally speaking the Branch performed very well in FY '79, even under the most adverse circumstances. As a service-oriented Branch whose "cradle-to-grave" involvement in the NIAID encompasses all grant programs, it is the goal of the GMB to more fully develop the partnership role that exists between the management specialist and their health-scientist counterpart, enhance the interaction between the management specialists and the grantee community, and to encourage and foster greater participation of the entire GMB staff in the "total" NIH grants management arena.

III. PROGRAM ANALYSIS AND EVALUATION BRANCH

The title "Program Analysis and Evaluation Branch" replacing "Review and Evaluation Branch" indicates a branch emphasis on development of an analytical base to develop reports and support the Office of the Director, NIAID Program Directors, and the NIAID staff data and information requests. A new management data base is being developed by branch personnel augmented by three one-time non-tenured personnel. All competing projects (996) and non-competing applications (1,812) will be entered into the new multi axis coding system, MACS.

The Branch serves as the focal point for management and budgetary data for Extramural programs; referral liaison with the Division of Research Grants, ADP Liaison with other Institutes and NIH, and reporting of non-program specific reports such as Prevention and Toxicology. The Branch was able to add age parameters to the revised PHS #398 (the grant application form) for inclusion in the MACS system and thus eliminate a search for this data.

A. Fiscal Management and Analysis Section

In FY 1979, the Fiscal Management and Analysis Section developed detailed operating plans for the use of all research and training funds and implemented the final plans. Awards were paid through both "Central" and "Program" accounts to assure payment of the highest quality research projects and programs of special interest to the aims of the Institute. Tables V and VI show final operating plans of EAP, IAID, and MID for contracts and grants in FY '79.

Due to the increase in the overall appropriation for research grants in FY '79, NIAID was able to pay about 43% of all research grant approvals and 61% of the approved career/academic award applications. In the absence of an appropriation for training and fellowships, this budget was subject to the conditions of a continuing resolution. Under this resolution, NIAID was able to fund 47% of approved individual fellowship requests and 24% of approved training applications.

Requests for fiscal reports increased significantly during the year to over three reports per week. Several studies were also undertaken by this Section to assess the impact of proposed changes in NIH policy. One such study, "Thinner Grant Applications on Longer Awards", was cited by NIH as an important factor in making their final decision.

TABLE V. NIAID
CENTRALLY CONTROLLED FUNDS
FY 1979 Operating Plan
(Dollars in thousands)

RESEARCH	IAID NO.	IAID FTE	IAID AMOUNT	MID NO.	MID FTE	MID AMOUNT	TOTAL NO.	TOTAL FTE	TOTAL AMOUNT
Commitment Base (Noncompeting):									
Regular projects (RO1, R22)	242		23,900	463		34,991	705		58,891
Young investigator awards (R23)	20		864	14		537	34		1,401
Career/academic awards (K's)	45		1,663	54		1,940	99		3,603
Scientific evaluation (RO9)	4		265	6		328	10		593
Minority biomedical support	1		125	--		--	1		125
RRB contracts	2		436	2		335	4		771
DNA contracts	--		--	4		369	4		369
MAN Lab (Georgetown)	--		--	1		929	1		929
Escrow against savings	--		350	--		550	--		900
Subtotal	314		27,603	544		39,979	858		67,582
Competing:									
Research projects (RO1, R22) -- (230)	133		11,022	339		26,027	472		37,049
Young investigator awards (R23)	4		162	4		161	8		323
Career/academic awards (K's)	14		495	9		278	23		773
Conference grants (R13)	1		18	6		89	7		107
International health (R21)	--		--	5		351	5		351
DNA contracts	--		--	1		24	1		24
Subtotal	152		11,697	364		26,930	516		38,627
Total, research.	466		39,300	908		66,909	1,374		106,209
TRAINING									
Commitment Base (Noncompeting):									
Individual (F32)	34	34	474	42	42	568	76	76	1,042
Institutional (T32)	30	139	2,063	38	189	3,005	68	328	5,068
MARC	1	1	22	--	--	--	1	1	22
Subtotal	65	174	2,559	80	231	3,573	145	405	6,132
Competing:									
Individual (F32)	27	27	383	44	44	613	71	71	996
Institutional (T32)	4	16	284	10	39	652	14	55	936
MARC	1	1	22	2	2	44	3	3	66
Subtotal	32	44	689	56	85	1,309	88	129	1,998
Total, training.	97	218	3,248	136	316	4,882	233	534	8,130
Total, centrally controlled funds	563	218	42,548	1,044	316	71,791	1,607	534	114,339

TABLE VI
NIAID
PROGRAM CONTROLLED FUNDS
FY 1979 Operating Plan
(Dollars in thousands)

	IAID		MID		TOTAL	
	No.	Amount	No.	Amount	No.	Amount
COMMITMENT BASE (noncompeting):						
Program projects (PO1).....	8	4,858	7	2,302	15	7,160
Asthma & Allergic Dis. Ctrs. (P50)...	9	1,498	--	--	9	1,498
CIRID (P50).....	4	1,198	--	--	4	1,198
ICMR (RO7).....	--	--	4	2,328	4	2,323
Regular research grants.....	7	488	11	887	18	1,375
R & D contracts.....	33	1,547	41	8,620[1/]	74	10,167
Escrow against savings.....	--	--	--	--	--	--
Subtotal.....	61	9,589	63	14,137	124	23,726
COMPETING:						
Program projects (PO1).....	2	1,076	3	1,051	5	2,127
Asthma & Allergic Dis. Ctrs. (P50)...	8	697	--	--	8	697
International Health (PO1).....	--	--	4	2,116[2/]	4	2,116
Regular research grants.....	--	--	1	81	1	81
R & D contracts.....	2	320	15	1,837	17	2,157
Other (uncommitted balance).....	--	517	--	--	--	517
Subtotal.....	12	2,610	23	5,085	35	7,695
Total, program controlled funds.....	73	12,199	86	19,222	159	31,421

1/ Includes $466,000 to cover ongoing flu contracts previously funded under NIIP.
2/ Special allocation from Director's Escrow for Secretary's Special Initiative (ICIDR).

B. Program Analysis Section

Since October 1978, the efforts of the Program Analysis Section have been almost exclusively directed toward an orderly and timely transition from the old scientific classification system (three-digit code) to the new multi-axis coding system (MACS). MACS is a complex four-digit hierarchial coding system consisting of four main axes:

Axis I - Organisms/Diseases/Study Area

Axis II - Approaches/Methodologies

Axis III - Anatomical Systems/Organs

Axis IV - Hosts

The scientific information in every active and pending grant application received in the NIAID, including all research and training mechanisms, are presently being reviewed and coded into the above system. Other areas of special interest which are also identified include:

1. Trans-NIH health problems (arthritis, blood-related diseases, bronchopulmonary disorders, digestive diseases, etc.);

2. Human subjects (clinical or non-clinical environment);

3. Special groups of human subjects (minors, pregnant women, etc.);

4. Age of subject;

5. Objectives of research/training (diagnosis, prevention, treatment, etc.);

6. R & D conducted at NIH (NIAID): Basic, Applied, and Development;

7. Entire spectrum of R & D activities at the NIH (NIAID): Science base, Applications, Transfer, and Training. Clinical trials, one part of Applications in SATT, is also included under Development. This information forms the basis of data which is developed into the Annual Inventory of Clinical Trials.

Analysis of the information captured by this newly developed intricate, multi-faceted coding system, coordinated with our assignment of all grants/grant applications to one of twenty-four program areas within the MIDP/IAIDP will be used to answer, more efficiently and accurately, repetitive and non-recurring types of queries from within NIAID (scientific management information for program planning and evaluation), NIH (e.g., Trans-NIH, BAD/SATT), members of Congress (problems of special legislative concern, e.g., Cystic Fibrosis, Sexually-Transmitted Diseases), other government agencies, and the public.

C. Data Control Section

The Data Control Section, using the NIH and Parklawn computer facilities, reports management, budgetary, and programmatic activities for the Extramural Activities Program. In addition, special and repetitive reports are provided for the Immunology, Allergic and Immunologic Diseases Program and the Microbiology and Infectious Diseases Program.

During fiscal year 1979, a new scientific information data base has been captured into NIAID's computer records and the system is nearing completion. With this implementation, computer generated reports are particularly explicit and will minimize selective interpretation in responding to requests for scientific data. The system allows for determining percentages of support in given areas, for retrieving TRANS-NIH and Programs of Institute Emphasis as identified by the Program staff involved. The new system has the capability of reporting areas of Basic, Applied, Developmental Research, and Scientific, Application, Training, and Technology Transfer in response to NIH concern.

The ongoing Contract system of data capture and retrieval of NIAID contract information services the contract office, budget office, O.D.'s office and continues to be well received.

IV. PROGRAM AND PROJECT REVIEW BRANCH

A. Introduction

In FY 1979, there was an enormous increase in the workload of the Branch, primarily involving scientific review by the Microbiology and Infectious Diseases Advisory Committee (MID). It was estimated that the MID review workload alone was at least 2.2 times the optimal workload of an average DRG Study Section. The heavy workload was a direct consequence of the release of numerous RFAs and RFPs by the MIDP in anticipation of a good budget in 1979 and a lean budget in 1980. In the March meeting alone a total of 68 program project, exploratory-developmental, and training grant applications were reviewed, as were 55 new competing contract proposals in response to 6 RFPs and one PL 480 proposal. It would have been humanly impossible for one executive secretary to have handled the review load. The Committee was therefore divided into subcommittees, each to review a bloc of grants or proposals, and then to meet on the fourth day as a full Committee for en bloc actions. This worked out reasonably well, with commendable teamwork and cooperation among review, grants management and contracts management, and other EAP staff. The Committee Chairman performed an outstanding job in chairing the two separate sessions of the full MID as well as two subcommittees. Still it is to be hoped that in the future more prudence be exercised in programming efforts that result in nearly unmanageable workloads for both EAP review staff and the Committee.

The Allergy and Clinical Immunology Research Committee and the Transplantation Biology and Immunology Committee both had reasonable review workloads and gave advice to the IAIDP on a variety of program issues. The AIRC advised on increased flexibility in acceptance of Allergic Diseases Academic Award (K07) applications. It also indicated that site visits should be conducted in a timely manner.

The increased workload of the MID impacted on the Review Services Unit, which serves the dual purpose of serving both the Council and the Review Committees, preparing the appropriate books of summary statements or applications and proposals for extramural staff and Council or Committee members.

Through all these, the Branch has been operating with a skeleton staff. A detailed manpower analysis of the Branch indicates that there is a very real need for an additional full-time (assistant) executive secretary as well as a clerk-typist to handle the workload in MID. The assistant executive secretary need not occupy a slot but may be a consultant. There is a need for another part-time worker in the Review Services Unit, or for one of the current part-time employees to be converted to full-time. Unless these needs are met in the immediate future, the quality of scientific review and the service offered by the Review Services Unit will suffer.

B. Allergy and Clinical Immunology Research Committee

The AIRC met three times during the period of November 1978 through September 1979, reviewing two program projects, four allergic disease center applications, seven training grant applications, and two Allergic Diseases Academic Award applications for a total first year requested amount of $1,914,788. In addition, an ad hoc contract proposal review was conducted on an amount of $2,265,794 for a two-year period. The Committee gave advice to the NIAID on policies regarding the Allergic Diseases Academic Award and on the program project mechanism. On the AADC program, the Committee advised on a much more flexible policy, which would allow multiple applications from multi-campus institutions and their clinical affiliates to be awarded. It is hoped that this liberal policy would move the program ahead. If the policy is accepted by Building 1, this would mean an escalation of the review activities of AIRC in the future.

Recommendations were made on program activities including food allergy, drug allergy, allergens, and fungal antigens. Concept clearance was given on the production and evaluation of horseradish-peroxidase-labelled antihuman globulin as an ELISA standard.

C. Transplantation Biology and Immunology Committee

The TIC met three times from November 1978 to July 1979, reviewing three training grants, one unsolicited contract proposal and four contract proposals submitted in response to an RFP for a total first year requested amount of $651,253. In addition, an ad hoc contract proposal review was conducted on a first year amount of $964,804. The Committee gave advice to the NIAID on the analysis and publication of the kidney transplant histocompatibility study data, organization and policies of the serum bank, and the formation of a hybridoma banking and distribution system. It gave recommendations on the development of RFPs for 1) design and evaluation of clinical tray configuration, 2) evaluation of alternative tissue typing techniques, and 3) absorption of anti-DR antisera, and RFAs for 1) post-transplant immunologic monitoring and 2) the role of non-MHC antigens in transplantation.

Dr. Harley Sheffield joined the Branch in FY '79 and has served as Executive Secretary of both the AIRC and the TIC. He, along with other professionals in EAP, has acted as executive secretary on ad hoc reviews of contract proposals within the IAIDP as well as the MIDP. The review workload of the AIRC/TIC is summarized in Table VII.

D. Microbiology and Infectious Diseases Advisory Committee

The Microbiology and Infectious Diseases Advisory Committee met four times from November 1978 through September 1979, reviewing 101 grant applications (39 program projects, 39 exploratory developmental grants, 23 training grants) for a first year requested amount of $24,964,364 direct costs; and 109 new competing, renewal, unsolicited, and sole source contract proposals for a total requested amount of $55,924,888

(Table I). Of the 39 program projects, two (STD program) were in fact reviewed by special review committees and site visited; the others (ICIDRs, mycology fungal disease research, arthropod-borne viral infections and tropical disease research units, and an unsolicited program project) were not site visited, but an ample number of consultants expert in the specific fields participated in the review and attended the meetings. Of the 109 contracts, 71 were reviewed by the full MID and 38 by ad hoc review groups with two committee members participating in each review. Of the 38 reviewed ad hoc, 12 were non-competing renewals and 26 were new competing proposals in response to one RFP of which one was from an MID Committee member.

Advice and concept clearance was sought from the full Committee on the following initiatives: Rocky Mountain Spotted Fever (epidemiologic studies, animal model), the role of Streptococcus viridans in bacterial endocarditis, penicillin prophylaxis by group B streptococcal sepsis in the newborn, and potentiation of immune responses to vaccines. Except for the last item, these have been implemented by RFPs and the proposals reviewed by the Committee.

E. Review Services Unit

For Council-related work, the Unit processed 2,045 applications and their respective summary statements for FY 1979. This compares with 2,049 applications and respective summary statements for FY 1978 (Table II). Although this difference is insignificant, Committee-related work has increased the volume of work for the RSU during this fiscal year, the applications processed increasing from 173 in 1978 to 222 in 1979, an increase of 28% (Table VIII). All grant applications for program projects, Centers, training and Allergic Diseases Academic Awards as well as all new contract proposals reviewed by the three NIAID Committees are processed by the Unit, with books made, reviewed for conflict of interest, and mailed to consultants as well as distributed to concerned staff. As was stated previously, the heavy workload of the MID impacted on the work of the Unit.

F. Clinical Trials

The reporting to DRG of clinical trials active in 1977 and 1978 was completed on June 5, 1979, far beyond the initial deadline set by DRG. Of the 128 trials reported, 51 were from the IAIDP, 65 from MIDP, and 12 from the intramural programs. The MIDP reported 14 new trials supported by contracts and 4 supported by grants. The IAIDP reported 4 new trials supported by grants and none supported by contracts. According to DRG, a formal publication of 1978 trials will be made (but not of the 1976 and 1977 trials, although printouts of data are and can be made available respectively). It is noted that the number of trials reported in 1976 was 142. The reasons for the seeming drop in number, from 142 to 128, could be the transitional quarter in 1976; or because of possible uneven reporting by program staff of grant-supported trials. Whereas in the past, a member of the Program Analysis Section screened the grants and identified clinical trials, in consultation with the NIAID Clinical Trials Coordinator, this year program staff have been asked to undertake this activity.

TABLE VII

REVIEW WORKLOAD, PPRB, 1978-1979

Category	1978 Number	1978 Amount	1979 Number	1979 Amount
AIRC/TIC/AD HOCS				
Grant Applications	59	$13,079,077	18	$2,409,163
Contract Proposals	45	13,550,639	24	5,835,232
Subtotal	104	-	42	-
MID/AD HOCS				
Grant Applications	49	12,594,364	101	24,964,364
Contract Proposals	57	16,342,567	109	55,924,888
Subtotal	106	-	210	-
CONTRACTS FOR OSD, OTHER	5	6,111,861	5	4,861,363
TOTAL	215	-	257	-

Grant amounts are first year direct costs only.
Contract amounts are total costs.

TABLE VIII

REVIEW SERVICES UNIT WORKLOAD

Category	1978	1979
For Council:		
Applications Received	2,049	2,045
"Pink Sheets" Received	2,049	2,045
For Committees:		
Applications Processed	108	119
Proposals Processed	65	103
Total Processed	173	222

V. RESEARCH RESOURCES BRANCH

A. Special Activities

ASM Exhibit - The Research Resources Branch handled arrangements for an NIAID exhibit at the American Society for Microbiology Meeting held on May 5-8, 1979, in Los Angeles, California. The RRB made the arrangements to take the Institute exhibit to Los Angeles while manpower used during the exhibit hours was supplied by the Office of Research Reporting and Public Response. Additional manpower was volunteered from the NIAID Extramural Activities Program, Microbiology and Infectious Diseases Program and from Intramural Laboratories. The exhibit was modified from the exhibit previously shown at ASM. An NIAID exhibit of different origin was repainted and new pictures and video tape installed to present the activities currently being carried out by NIAID. The exhibit was well received and gave many visitors knowledge of the various activities being supported by the Institute.

Dr. Robert Byrne has continued to serve as Branch Chief with Sylvia Cunningham responsible for management of operations including the distribution and cataloging of the microbial, allergen and some of the immunological reagents.

The need for well characterized reference reagents as an adjunct to research is well recognized, and in support of the concept, the Research Resources Branch (RRB) conducts a program which distributes a wide range of reagents to research scientists. Reagents for which a critical need exist are identified and arrangements for their production are made by the pertinent Institute Program area. The RRB then arranges for the packaging of the bulk products, catalogs the item and distributes the reagent with the information and technical advice on reagent characteristics and use. The Branch is also sponsoring a contract for the development of a procedure for the processing and packaging of recombinant DNA Vector-Host Systems. In addition, the Branch assists with the contract for the establishment of a Plasmid Reference Center.

B. Asthma and Allergic Diseases Program-Allergen Reagents

A procurement contract has been awarded to the American Type Culture Collection to process the minor determinant penicillin products. The RRB has made all the arrangements to have these products packaged and lyophilized. The actual processing is expected to begin the week of July 30; it is expected that approximately 4 months will be required to complete all lots of material. After an IND is submitted, these products will be supplied to approximately six contractors who will be responsible for conducting clinical trials to ascertain the penicillin sensitivity of patients.

The contract with Johns Hopkins University for the preparation of polyvalent ryegrass antiserum was completed this year. This material

has been processed by the American Type Culture, undergone certification testing by Mayo Clinic and is now available for distribution.

Although the contract located at University of Pennsylvania for the establishment of an allergic dog colony is sponsored by IAIDP, the Chief, RRB has continued to serve as a project officer on this contract. The contract has been highly productive in the breeding of dogs and is ahead of schedule in producing progeny of atopic breeding stocks.

In addition to the activities listed above, the Branch has continued to distribute allergenic products such as Ragweed Antigen E, K, and RA3, Ryegrass I, II, and III and venoms of honey bee, yellow hornets, white-faced hornets, yellow jackets, paper wasps and hymenoptera venom diluent; these venom products are available for distribution under an IND as well as for in vitro use.

C. Immunological Reagents and Resources

The new contract awarded to Flow Laboratories during FY 1978 for the Maintenance and Breeding of Rabbits of Known Genotype for Use in Immunological Studies has continued to serve a useful purpose in maintaining a colony of 500 specially bred rabbits for use in NIAID immunological studies. In addition, these facilities provide reference quantities of reagents and limited numbers of rabbits to other responsible investigators doing immunological studies. During this period, seven investigators have been supplied with a total of 18 rabbits; twenty-six shipments of rabbit antisera have also been made to other investigators.

During this period, the rabbits had to be moved from their building in Rockville to make room for the new Metro station. The rabbits are currently being housed in Fairfax, Virginia, in a building being leased by Flow from Litton-Bionetics. Flow expects their new animal building which is being built in McLean, Virginia, to be completed in September; the animals will be moved again when the new building is ready for occupancy.

D. Microbiology

During the latter part of FY 1977, two contracts were awarded and have continued during FY 1979. The University of Alabama is performing research and developmental work to determine optimal conditions for the preservation, retrieval, storage and distribution of fragile bacterial host strains. Three basic methods of storing have been studied, i.e., preservation in 1) paraffin-sealed agar stabs, 2) by freezing in peptone-glycerol broth and 3) by lyophilization. Results to date indicate that lyophilized material stores better at -30°C or -70°C than at 4°C or room temperature. The peptone-glycerol broth freezing method appears to cause some reversion frequencies of some products. This problem is now being carefully analyzed; similar

studies are being conducted on the lyophilized materials to determine if the reversion frequencies are also occurring in these samples. The MIDP is sponsoring the Stanford University contract for the establishment of a plasmid reference center. During the year a modification was made to the contract work scope to allow the contractor to continue storing and shipping the bulk cultures. RRB will therefore, not be responsible for the packaging, storing and distribution of the strains collected under this project. However, backup set of these strains are being maintained by RRB (to date 90 sets have been received for storage). In addition, RRB will assist with the publication and printing of the Catalog of Plasmid Strains once a suitable format has been developed. To date, the format for the strains data sheet has been revised three times.

E. Research Resources - Research Reagents

While responsibility for the actual production of reagents is with the various Institute program areas, RRB has continued to sponsor certain service type contracts which serve the needs of the various program elements.

The Ohio State University has continued to serve as the viral reagent testing laboratory. In addition to verifying feedback data from reagent users concerning product viability, the contractor has verified certain results obtained by the American Type Culture Collection on the reagent transfer contract. During this year, the contractor has conducted tests on 15 rhinovirus seeds and antisera, 4 enterovirus seeds and has confirmed the contamination of reovirus type 1 with SV40. The contractor is now initiating cross-neutralization testing on adenovirus types 10 and 13 to see if he can verify a report from a reagent user that our adenovirus type 13 rabbit serum neutralizes adenovirus type 10.

After a category of reagents has become established, and the area of research that it supports is well defined, it is of questionable value for RRB to continue storing and distributing that category of reagents for a prolonged period of time. During FY 1975, it was determined that four viral reagent groups fall into this category. RRB therefore, entered into a five year contractual arrangement with the American Type Culture Collection (ATCC) to transfer the enterovirus, adenovirus, rhinoviruses and arboviruses reagent collections to the ATCC whose prime function is to store and distribute these types of reagents.

Under the contractual arrangement with ATCC, samples of each reagent type will be preserved as a museum function. The viability or homologous antibody activity of a parallel collection of reagents will also be assayed. After each viral group has been assayed and the results reported and reviewed, RRB will discontinue that reagent group from its catalog with a notation that they are available from ATCC and all remaining stock of that group of reagents will be transferred

to ATCC for its custodianship. NIH scientists and NIAID contractors will be provided reagents without cost until RRB supply is depleted.

During the first four years of this project, 50 samples of each reagent type for the four viral reagents groups have been transferred to ATCC for the museum function. The assay of the enterovirus, adenovirus and rhinovirus groups has been completed; these three groups have now been transferred to the ATCC. During FY 1979, ATCC has primarily been involved in the testing of the arbovirus reagent collection which is the last of the four collections to be re-assayed before being transferred to ATCC. The schedule for assaying and transferring reagents originally prepared in 1975 is still as projected; it is planned that the assay of arbovirus reagents will be completed during FY 1980 with the inventory of arbovirus reagents being transferred to ATCC upon completion of review of the data.

The Branch has also been involved in assisting with plans to have the rotavirus reagents labeled and packaged. These materials are being prepared under an Interagency Agreement with Plum Island, USDA which is being supported by MIDP.

The Branch has also given assistance with the preparation of other contract documentation, such as work-scope, reporting requirements, evaluating criteria, etc., to have additional arbovirus reagents prepared. During the latter part of FY 1979, an interagency agreement will be made with the USDA for the preparation of an arbovirus grouping fluid comprised of African swine fever, bovine ephemeral fever, African horse sickness and Rift Valley fever. During FY 1980, requests for proposals will be issued to have other arbovirus reagents prepared. The reagents resulting from these projects will ultimately be received by RRB for processing (if necessary), cataloging and distribution.

Prior years of intensive work have resulted in completed work on the enterovirus, adenovirus, rhinovirus, myxovirus and the agents and antigens of hepatitis A & B. In most cases, seed virus preparations and corresponding antisera are now available for most of the viruses of public health interest. The reagents which have resulted from the various projects have been most useful, particularly the hemagglutinins, neuraminidases, ribonucleoproteins and the matrix proteins of the influenza viruses of man and animals. Since completion of this production effort in FY 1978 and listing in the 1978 - 1980 Catalog of Research Reagents, these materials have become a very popular distribution item. Information received thus far from the reagent users indicate that these materials are very valuable.

F. Molecular Anatomy Program

The Molecular Anatomy Program (MAP) during FY 1979 has been evaluating for safety and efficacy in human vaccine trials, subviral forms of hepatitis B surface antigen (HBsAg), purified from the plasma of chronic carriers and inactivated. Thus far, limited studies in volunteers have shown the vaccines to be free of infectious hepatitis B virus and local

and systemic side reactions. MAP is currently evaluating different vaccine formulas and inoculation schedules in volunteers to optimize the humoral anti-HBs response. Four vaccine lots have been prepared from the original inactivated HBsAg/adw preparation; (1) untreated, aqueous, (2) untreated, alum-adsorbed, (3) ether-tween 80 treated, aqueous, and (4) ether-tween 80 treated, alum-adsorbed. The four lots passed safety tests and are currently being evaluated in volunteers at the NIH. A large lot (1000 doses) of the untreated, alum-adsorbed vaccine and an alum placebo were also prepared for more extensive testing by Dr. Hollinger at Baylor Medical School under an NIAID contract; the vaccine and placebo lots are currently on safety tests.

The hybridoma technology offers great promise for analyzing the antigenic determinants of HBsAg. Hybridomas from spleen cells of Balb/c mice immunized with HBsAg/adw and mouse myeloma cells, P3 X 63Ag8, have been developed.

Many laboratories are now engaged in efforts to detect antigen-antibody systems related to the non-A, non-B hepatitis agents. MAP has involved in defining the delta antigen and its antibody (δ/anti- δ). This specificity is found in carriers of HBsAg and appeared to be related to the HBV system. A sensitive RIA for anti-δ has been developed and found predominately in chronic HBsAg carriers, although a few asymptomatic carriers were also positive for anti-δ; low and transient titers of anti-δ were found in acute HBV hepatitis patients. The evidence to date indicates that δ represents a marker of an agent, not HBV, which requires HBV infection to provide certain helper functions for its replication.

The eastern woodchuck (Marmota monax) has an HBV-like virus and this animal may represent an inexpensive model of virus-induced liver disease including hepatocellular carcinoma. MAP has examined approximately 50 wild-caught animals and based on endogenous DNA polymerase activity and virus-like particles, approximately 30% are chronically infected with this virus. We showed that the woodchuck virus cross-reacts with HBV in both HBsAg and HBcAg components. Two of the carrier woodchucks have recently been diagnosed with hepatocellular carcinoma. Further evaluation of the woodchuck as a model of HBV infection and disease will continue.

Efforts in respiratory syncytial virus research have concentrated on the isolation and identification of surface antigens of the virus. The known instability of the virus has been a major problem. However, 1 M $MgSO_4$ has been very effective in stabilizing the infectivity of the virus throughout the purification procedures. Large-scale isolation of virus under stabilizing conditions are now in progress. Several lines of mouse Balb/c cells have been established that are persistently infected with RSV. These cells produce low levels of infectious virus, contain large amounts of ribonucleoprotein and express RSV-specific antigens on the cell membrane. These cells will be useful in the development of murine hybridomas for the production of monoclonal antibodies to surface antigens of the virus.

G. Processing and Distribution

In addition to the various program elements detailed above, the Research Resources Branch also distributes coronaviruses, herpes viruses, interferons, mycoplasmas and reoviruses.

Due to the limited number of reagents to be processed during FY 1979, there was not a formal research and development contract to handle this activity. However, the American Type Culture Collection did process four (4) mycoplasma seeds and one antiserum lot by the purchase order mechanism.

The Research Resources Branch reagent collection (exclusive of the viral reagents transferred to ATCC) now consists of over 650 individual reagents. The repository and distribution contract remains at Flow Laboratories. During this period, the repository activity was moved from its Rockville, Maryland, to a new building in McLean, Virginia. A tabular record of distribution by this facility since FY 1972 follows:

DISTRIBUTION OF VIRAL, MYCOPLASMAL, AND ALLERGEN REAGENTS

Fiscal Year	Total Transactions*	Total amps. & vials Distributed
1972	572	21,801
1973	575	19,181
1974	500	9,932
1975	592	6,751
1976	762	10,188
1976 (TQ)	192	3,126
1977	613	7,633
1978	605	6,851
1979 (3/4 year)	561	6,907

*Does not include the shipments made for testing and packaging purposes and reagents transferred to the American Type Culture Collection.

In addition to the shipments tabulated above, Flow has also made 141 shipments for DAB, 28 shipments consisting of whole rabbits or rabbit serum and 30 interferon shipments for the National Cancer Institute. All of this was conducted under the NIAID contract with the NCI contributing funds to NIAID for their activities. In addition, American Type Culture Collection has made the following shipments under the reagent transfer contract:

(U.S. Investigators and WHO Laboratories)

	# of Shipments	# of Ampoules
FY 1978	96	1,622
FY 1979 (3/4 year)	87	1,068

During this year, the printing of the new 1978 - 1980 Catalog of Research Reagents was completed by the Government Printing Office. Over 1900 of the 2200 copies printed have been mailed out to reagent users.

The Branch has continued its efforts to publicize the availability of these valuable reagents to the scientific community. The availability of reagents was incorporated into the NIAID exhibit which was shown at the American Society for Microbiology meeting held in Los Angeles, California.

The following two (2) tables describe the distribution of reagents by groups and also by institutional affiliation during the first three quarters of FY '79.

RESEARCH RESOURCES BRANCH
DISTRIBUTION OF SPECIFIC VIRUS GROUPS
FY 1979 (3/4 year)
OCTOBER 1, 1978 - JUNE 30, 1979

# of Items Avail. (% of Inventory)	Virus Group	SHIPMENTS # of	SHIPMENTS % of	AMPOULES # of	AMPOULES % of # Distribution
9***(1.78)	Adenovirus	3	.5	18	.3
17 (3.36)	Allergen	81	14.4	1345	19.5
224 (44.27)	*Arbovirus	43	7.7	647	9.4
4 (.79)	Coronavirus	3	.5	9	.1
20 (3.95)	Recombinant DNA	27	4.8	244	3.5
20 (3.95)	**Enterovirus (Typing Pools)	80	14.4	2294	33.2
18 (3.56)	Hepatitis	40	7.1	240	3.5
18 (3.56)	Herpes	2	.4	3	.1
5 (.99)	Interferon	133	23.7	237	3.4
86 (17.00)	Mycoplasma	49	8.7	837	12.1
79 (15.60)	Myxovirus Paramyxovirus and related	63	11.2	575	8.3
6 (1.19)	Reovirus	8	1.4	37	.5
	Combinations	29	5.2	421	6.1

Figures based on 561 shipments and 6907 ampoules shipped (excludes testing, packaging and reagents transferred to ATCC).

*Included in this figure are 11 shipments of Arbovirus Grouping fluids totaling 201 ampoules.

**Included in this figure is 1 shipment to a WHO Laboratory totaling 1130 ampoules.

***Seed and antisera for adenovirus types 1 thru 31 have been transferred to the ATCC.

RESEARCH RESOURCES BRANCH
FY 1979 SHIPMENTS
OCTOBER 1, 1978 - JUNE 30, 1979

Type of Organization	Shipments to Grantees	Items Shipped	Shipments to Contractors	Items Shipped	Other Shipments	Items Shipped	Total Shipments	Total Items
Universities	42	239	8	46	113	1200	163	1485
Commercial	10	175	2	47	30	269	42	491
Hospitals	16	150	1	3	45	449	62	602
Foundations (Non-profit)	6	27	1	10	17	62	24	99
State or City Health Labs	3	31	0	0	15	106	18	137
Other Federal Agencies	0	0	0	0	18	241	18	241
NIH	0	0	0	0	65	542	65	542
Foreign	0	0	0	0	162	1742	162	1742
WHO	0	0	0	0	2	1212	2	1212
Miscellaneous	1	55	0	0	3	218	4	273
Reagent Testing	0	0	0	0	19	0	19	0
Bulk for Packaging	0	0	0	0	2	108	2	108
Discard	0	0	0	0	2	984	2	984
ATCC Transfer	0	0	0	0	0	0	0	0
TOTALS	78	677	12	106	493	7133	583	7916

H. Individual Contract and Agreement Tabulation

PROGRAM: IMMUNE SYSTEM AND DISEASE - REAGENTS AND RESOURCES BRANCH: RRB

Principal Investigator Contract Number Contractor FY '79 Obligations	Objectives and Findings
Sullivan, Rudolph R. AI 82565 Flow Laboratories $158,700	To maintain a genetically bred rabbit colony of known genotype for use in immunologic investigations. A maximum of 500 adult rabbits will be bred and maintained by expert veterinary care. This is for support of high priority research on genetic control of the immune response and to supply reagents and rabbits to other investigators.

Individual Contract Agreement Tabulation

PROGRAM: INFECTIONS - VIRAL REAGENTS BRANCH: RRB

Principal Investigator Contract Number Contractor FY '79 Obligations	Objectives and Findings
Hughes, John R. AI 62513 Ohio State University $17,313 (FY 1978 Funds)	This laboratory serves as a shelf-life viral reagents testing laboratory for members of the enteroviruses, rhinovirus, adenovirus, myxovirus and herpevirus groups. This contract will ensure the integrity of these reference standard reagents by assessing the potency and purity of viral reagents after long term storage. This contract will terminate this year.

Individual Contract Agreement Tabulation

PROGRAM: INFECTIONS - MOLECULAR ANATOMY PROGRAM BRANCH: RRB

Principal Investigator Contract Number Contractor FY '79 Obligations	Objectives and Findings
Gerin, John AI 82572 Georgetown University $928,952	The major objective is to develop and apply methods of isolating viruses and viral antigens and the application of those techniques to research on associated diseases. During FY 1979, this laboratory has been involved in purifying and inactivating subviral forms of hepatitis B surface antigen (HBsAg) from the plasma of chronic carriers. These materials are currently being evaluated for safety and efficacy in human vaccine trials. Limited studies in volunteers have shown the vaccines to be free of infectious hepatitis B virus and local and systemic side reactions. The contractor has also developed hybridomas from spleen cells of Balb/c mice immunized with HBsAg/adw and mouse myeloma cells, P3 X 63Ag8. He also has been involved in defining the delta antigen and its antibody (δ/anti-δ). This specificity is found in carriers of HBsAg and appears to be related to the HBV system. Evidence to date indicates that δ represents a marker of an agent, not HBV, which requires HBV infection to provide certain helper function for its replication. The contractor is also now involved in the large-scale isolation of RSV under stabilizing conditions which have been developed.

Individual Contract Agreement Tabulation

PROGRAM: INFECTIONS - PROCESSING AND DISTRIBUTION BRANCH: RRB

Principal Investigator Contract Number Contractor FY '79 Obligations	Objectives and Findings
Stanley, Thomas AI 82542 Flow Laboratories $184,506 (MID) $276,758 (IAIDP)	When the reagents program was given the responsibility for production of reference reagents, other responsibilities were also included, i.e., the development and maintenance of appropriate means to receive, process, store and distribute these research materials. The contractor fulfills these responsibilities. During the past year, the contractor has been supplying the storage and shipping services for the RRB, Developmental Applications Branch, the Enteric Diseases Branch and the NCI. As a result of these combined activities, the contractor has made 2548 shipments totaling 106,614 vials + 38,670 trays and 570 shipments of bulk material. Approximately 95 incoming shipments have been handled.

Individual Contract Agreement Tabulation

PROGRAM: INFECTIONS - PROCESSING AND DISTRIBUTION BRANCH: RRB

Principal Investigator Contract Number Contractor FY '79 Obligations	Objectives and Findings
Clark-Curtiss, Josephine AI 725333 University of Alabama $171,657 (FY 1977 funds) Contract awarded for a 3-year period	This contractor is determining the optimal processing, packaging and storage conditions for vector-host systems for recombinant DNA molecule research. To date, the contractor has investigated various methods of preserving these materials. The methods employed to date include 1) preservation in paraffin - sealed gelatin slabs 2) preservation by freezing in peptone - glycerol, preservation by lyophilization. In addition, a study is being conducted comparing the effects of various storage temperatures.

Individual Contract Agreement Tabulation

PROGRAM: INFECTIONS - PROCESSING AND DISTRIBUTION BRANCH: RRB

Principal Investigator Contract Number Contractor FY '79 Obligations	Objectives and Findings
Donovick, Richard AI 75-2526 American Type Culture Collection $149,823	After a category of reagents has become established and the area of research that it supports is well defined, it is of questionable value for RRB to continue storing and distributing that category of reagents. Therefore, in FY '75, RRB entered into a cooperative enterprise with ATCC to transfer the enterovirus, arbovirus, rhinovirus and adenovirus reagent collections to the ATCC for their custodianship and distribution; complete transfer of the four viral reagent groups will be accomplished over a five-year period. During the first three years of the contract, 50 samples of each reagent type for the four viral reagent groups have been transferred for museum storage purposes. Under the terms of the contract, each of the four collection of reagents is to be assayed for viability or homologous antibody before the complete transfer is made, therefore, the contractor has been assaying the enterovirus, adenovirus, rhinovirus groups during the initial period. The assay of these groups has been completed, the test results have been reviewed, and the total enterovirus, adenovirus, rhinovirus collections have been transferred to ATCC. The contractor is now performing the required testing on the arbovirus reagents.

CONTRACT PUBLICATIONS

Gerin, J. L. and J. W. -K. Shih. Structure of HBsAg and HBcAg. In: Viral Hepatitis (Eds., G. N. Vyas, S. N. Cohen and R. Schmid). pp. 147-153. Franklin Institute Press, Philadelphia. 1978.

Feinstone, S. N., Y. Moritsugu, J. W. -K. Shih, J. L. Gerin and R. H. Purcell. Characterization of hepatitis A. virus. In: Viral Hepatitis (Eds., G. N. Vyas, S. N. Cohen and R. Schmid). pp. 41-48. Franklin Institute Press, Philadelphia. 1978.

Purcell, R. H. and J. L. Gerin. Hepatitis B. vaccines: A status report. In: Viral Hepatitis (Eds., G. N. Vyas, S. N. Cohen and R. Schmid). pp. 491-505. Franklin Institute Press, Philadelphia. 1978.

Dreesman, G. R. and J. L. Gerin. Summary of workshop on structure of hepatitis viruses. In: Viral Hepatitis (Eds., G. N. Vyas, S. N. Cohen and R. Schmid). pp. 645-648. Franklin Institute Press, Philadelphia. 1978.

Shih, J. W. -K., G. Hess, P. M. Kaplan and J. L. Gerin. The polypeptides of HBV (Dane) particles. In: Viral Hepatitis (Eds., G. N. Vyas, S. N. Cohen and R. Schmid). pp. 705. Franklin Institute Press, Philadelphia. 1978.

Shih, J. W. -K., M. Rizzeto, C. Langer and J. L. Gerin. 1979. Type B hepatitis: Characterization of delta antigen and the development of radioimmunoassays for delta and anti-delta. Abstr., Annl. Mtg. Amer. Soc. Microbiol. S(H)80.

Rizzetto, M., D. Gocke, J. Shih, G. Verme and J. L. Gerin. 1979. A solid-phase radioimmunoassay for antibody to the hepatitis B associated delta antigen: Incidence of anti-delta in HBsAg carriers. Abstr., Annl. Mtg., Amer. Soc. Microbiol. S(H)14.

Rizzetto, M., J. W. -K. Shih and J. L. Gerin. 1979. Characterization of the hepatitis B virus (HBV) associated delta antigen: Incidence of anti-delta in HBsAg carriers by a solid-phase radioimmunoassay (SP-RIA). Fed. Proc. 38(3).

Hess, G., J. W. -K. Shih, J. L. Gerin, R. H. Purcell and K. H. Meyer zum Bueschenfelde. Hepatitis B. antigen: Lack of relationship to liver-specific protein (LSP). J. Med. Virol. (In Press).

Parrott, R. H., H. W. Kim, C. D. Brandt, M. O. Beem, L. Richardson, J. L. Gerin and R. M. Chanock. Respiratory Syncytial virus. In: Diagnostic Virology (Ed., E. Lennette). (In Press).

Hess, G., J. W. -K. Shih, W. Arnold, J. L. Gerin and K. H. Meyer zum Bueschenfelde. Demonstration and partial characterization of 22 nm HBsAg and Dane particles of subtype HBsAg/adw. J. Immunol. (In Press).

Shih, J. W. -K., G. Hess, P. M. Kaplan and J. L. Gerin. Characterization of hepatitis B virus (Dane particle). J. Virol. (submitted).

Rizzetto, M., J. W. -K. Shih, D. J. Gocke, R. H. Purcell, G. Verme and J. L. Gerin. Incidence and significance of antibodies to delta antigen in hepatitis B infection. N. Engl. J. Med. (submitted).

Moritsugu, Y., J. W. -K. Shih, S. M. Feinstone, J. L. Gerin and R. H. Purcell. The hepatitis A virion: Polypeptides of hepatitis A virus obtained from stool of a naturally infected patient. J. Virol. (submitted).

Shih, J. W. -K., P. J. Cote, Jr. and J. L. Gerin. Production of monocolonal antibodies against hepatitis B surface antigen by somatic cell hybrids. AABB Annl. Mtg. (submitted).

Rizzetto, M., R. H. Purcell and J. L. Gerin. Antibody to the hepatitis B virus-associated delta antigen in hemophiliacs: Evidence for parenteral transmission of delta through superinfection. AABB Annl. Mtg. (submitted).

Purcell, R. H., R. A. Johnson, V. J. McAuliffe, J. L. Gerin, W. T.. London, E. Nordenfelt and E. Helgstrand. Effect of antivirals on markers of HBV infection in chimpanzees. Lancet (submitted).

OFFICE OF THE SCIENTIFIC DIRECTOR, NIAID
1979 Annual Report

Table of Contents

SUMMARY OF PROGRAM

Laboratory and Clinical Research, NIAID

October 1, 1978 through October 1, 1979

Office of the Scientific Director

The Annual Report of the Intramural Research Program contains individual summaries of research projects in the twelve laboratories which constitute the research component of the National Institute of Allergy and Infectious Diseases. Administrative responsibility for the Intramural Research Program resides in the Office of the Scientific Director (OSD). The OSD has been reorganized during the period of the past twelve to eighteen months in order to provide for more effective management. The reorganization also allowed for distribution of responsibility and authority to all working levels.

Mr. Charles Criswell has served as the Administration Officer in charge of Intramural Operations. Three Administrative Assistants have been appointed, each of whom report to his office. These assistants, Mrs. Helen Bednarek (Buildings 5 and 8), Mrs. Cathy Sabo (Building 7) and Mrs. Bea McKinley (Building 10) have assumed responsibility for all personnel, budgetary and other administrative matters in each of their respective areas. Assistance has been provided to each of the Administrative Assistants through the assignment of one procurement technician and one clerk typist to perform administrative functions for all laboratories in each of the geographic areas. Also reporting to Mr. Criswell is Mr. Carter Smith who serves as Head of the Animal Care Section which currently consists of 19 animal caretakers among which two serve as team leaders. The animal care operations at the Bethesda campus have been the direct responsibility of this Animal Care Section. During the past year a new office has been added in OSD. The Editorial Office headed by Mrs. Betty Sylvester (Editor) is now assisted by two editorial assistants. This office utilizes the most modern of word processing equipment to provide support primarily for the production of manuscripts. Currently, they have assumed a workload sufficient to take care of the needs of four of the in-house Bethesda campus laboratories. It is hoped that sufficient resources will be available to expand their services to provide support to all intramural laboratories.

A new office of Special Assistant to the Scientific Director has been established to provide for direct responsibility for Safety and EEO programs in the IRP. Dr. Katherine Cook Jaouni has been appointed to this position and has performed in a remarkably good manner. Of particular importance was the development of a Minority Biomedical Sciences Program which brought forty young undergraduate minority scientists to Washington for a meeting in the spring of 1979. From amongst this group, ten were selected to participate in research activities of the Intramural Program in the summer of 1979. The program was outstandingly successful and will be the forerunner of similar programs to be developed in future years.

The Rocky Mountain Laboratory in Hamilton, Montana was reorganized this year

with the plan finally approved formally on March 16, 1979. This resulted in the Division of this large laboratory complex at RML into three scientific laboratories, the Laboratory of Persistent Viral Diseases, the Laboratory of Microbial Structure and Function and the Epidemiology Branch. In addition, the Administrative activities of RML were assigned to the Operations Branch - RML. Mr. Robert Steiner serves as Chief of the Operations Branch. Currently, three acting Lab Chiefs are operating the three new laboratory facilities at RML and a process of search and selection is underway to identify permanent laboratory chiefs for these three new laboratory activities. The reorganization of RML into three scientific areas of research will allow for a focusing of research endeavor and allow for the build-up of research expertise at RML. It is anticipated that up to 15 new scientists will be added to the Laboratory. Because of restriction in total number of personnel allowed within the budget for assignment at RML, this will require a concomitant reduction of support personnel. It is anticipated that loss of support personnel will be offset through the use of local contracts to provide needed services to the Rocky Mountain Laboratory.

The Laboratory of Viral Diseases was divided during this year in order to allow Dr. Wallace Rowe, Chief of LVD, to direct his energies to the areas of personal concern and research interests. The Laboratory had been difficult to manage because of the division of its laboratory members into two geographic areas. The majority of the members of the Staff located in Building 7 will be retained in LVD and work directly with Dr. Rowe. The members of the staff which had been located in Building 5 have temporarily been assigned to the Office of the Scientific Director.

During the past year, several Intramural Laboratories were reviewed by the Board of Scientific Counselors. They reviewed the Laboratory of Parasitic Diseases, the Laboratory of Infectious Diseases, the Laboratory of Viral Diseases, the Laboratory of Streptococcal Diseases and the Laboratory of Biology of Viruses. In each instance, up to six Ad Hoc Consultants were added to the Board of Scientific Counselors in order to provide greater expertise in the review of each of the laboratories. Each investigator, including both tenured and nontenured investigators in each laboratory submitted a written report of their ongoing and future work. At the time of the on-site visit by the Board of Scientific Counselors and its Ad Hoc members, there was a general presentation of Laboratory activities but, in addition, each investigator, both tenured and nontenured, was interviewed for a period of an hour by at least two members of the Board. This format has allowed an intensive evaluation of overall program and individual contributions to programs within each of the laboratories. The Board of Scientific Counselors has been instrumental in providing useful suggestions and recommendations following each of their visits to the laboratories.

The scientific accomplishments of the Intramural Program have been exciting. Many members of the Staff have received significant awards and recognition from their peers and from the appropriate scientific organizations. Perhaps the most significant award of all was the receipt of the Paul Ehrlich prize with its sizeable cash award given to Dr. Wallace Rowe for his work in the fields of viral leukemia and immunogenetics.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-A1-00013-16 OSD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Studies of Viral Antigens in Virus-induced Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI	:	Andrew M. Lewis, Jr.	OSD, NIAID
Other	:	Hubert W. Gerry	Staff Fellow, NIAID

COOPERATING UNITS (if any)
Charles Kirkpatrick (NIAID); Arthur Levine, Cephas Patch, Alan Rabson (NCI); Heiner Westphal, (NICHHD) Robert Martin (NIAAMD)

LAB/BRANCH
Office of the Scientific Director

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.5	2.75	3.75

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) The initial objectives of this project were to use human adenoviruses and adenovirus - SV40(Ad2-240) recombinants as tools to study the genetics of DNA tumor viruses, to define the role of viral genes and viral antigens in viral oncogenesis and to study the biology of Ad2-SV40 recombinants. Due to the lack of proper containment facilities in NIAID between January 1973 and May 1978, there has been a 5-year disruption (see past reports) in the major thrust of this project. During this interval, a study of Ad2-SV40 recombinants associated tumor induction by these agents with the incorporation into the adenovirus - 2 chromosome of a specific segment of SV40 DNA. To begin to understand the mechanism by which this SV40 DNA segment conveys oncogenicity to these recombinants, it was necessary to understand why Ad2 was nononcogenic for hamsters. Since Ad2 will transform hamster cells in tissue culture, we initiated a study of the oncogenic properties of Ad2 transformed cells. The results thus far lead us to suspect that unrecognized host immune mechanisms in the hamster reject Ad2 transformed cells and transformed cell-induced tumors. Such a concept suggests that the presence of a specific segment of the SV40 genome might induce functions in hamster tumor cells that interfere

with the rejection process. Future studies will be directed toward more carefully defining these concepts.

Project Description

Major Findings: Oncogenicity of SV40 deletion mutants that induce altered 17K t proteins

The nondefective hybrid, $Ad2^{+}ND_4$, which contains the segment of the SV40 genome between map position 0.11 and 0.59, induces tumors in hamsters after inactivation by UV light (see 1977-1978 Report). Since nonhybrid Ad2 and the $Ad2+ND_2$ hybrid which contains the segment of the SV40 genome between map position 0.11 and 0.43 do not induce tumors after UV inactivation, these results imply that the segment of the SV40 genome between 0.43 and 0.59 is required for tumor induction. To more carefully define the region of the SV40 genome associated with tumor induction, we studied the tumor-inducing capacity of a number of SV40 mutants containing deletions of the early region between map position 0.54 and 0.59. These mutants induce a normal 92,000 dalton T protein, but induce altered 17,000 t proteins. Twenty to 90% of hamsters inoculated as newborns with SV40 mutants Δ883, Δ885, Δ886 Δ890, Δ2001 and Δ2005 developed tumors after a 6 to 12 month latent period. For this study, SV40 wild-type virus produced tumors in 90% of inoculated hamsters within 8 months. The differences in the latent period prior to tumor development by SV40 compared with the SV40 mutants was statistically significant. Together with the studies on tumor induction by the $Ad2+ND_4$ hybrid, these studies imply that the DNA sequences comprising the initial portion of the early SV40 genome between map positions 0.65 and 0.54 are not essential for tumor induction in hamsters. Although the 17 k t-protein which is encoded by the SV40 region between map position 0.65 and 0.54 is not essential for tumor formation, it does appear to reduce the latent period prior to tumor development.

Host response to adenovirus 2 transformed hamster embryo cells

The finding that a segment of SV40 DNA, by recombining with the nononcogenic Ad2 genome, conveyed tumor-inducing properties to the $Ad2^{+}ND_4$ raised questions about the mechanism by which this change to oncogenicity was effected. Others have shown that Ad2 transformed rat cells were incapable of establishing tumors in immunocompetent rats. Since there appears to be little or no difference between the *in vitro* transforming efficiency of the $Ad2^{+}ND_2$ and $Ad2^{+}ND_4$ recombinants and nonhybrid A2, it seemed reasonable to assume that the change toward oncogenicity could be a reflection of the manner in which cells transformed *in vivo* by hybrid and nonhybrid virions interacted with the host immune system. As there was little data about the tumor inducing capacity of Ad2 transformed hamster cells, it was necessary to examine this problem before the recombinant could be considered.

Ad2 inactivated by UV light was used to transform LSH hamster embryo cells. Evaluation of transformed cell lines produced by Ad2 (strain adenoid 6) or isolates of Ad2 obtained from children in Washington, D. C., or West Bengal, India, showed that 13 of 15 lines induced tumors when injected into newborn inbred LSH or randomly bred NIH hamsters (10^7 cells/hamster) with a median tumor incidence in syngeneic newborns of 65% (range 8 to 100%). Six out of 6

of these cell lines did not induce tumors when 10^7 cells were injected s.c. into weanling animals over 21 days old. In contrast to the Ad2-transformed lines, similarly derived cell lines transformed by Ad12 or SV40 uniformly produced tumors in weanlings following s.c. inoculation of 10^7 cells. Transplantation of tumors from animals injected as newborns with Ad2-transformed cell lines to other newborns was readily accomplished in 639 of 640, (99.8%) inoculated newborns. However, only 53 of 467 (11.3%) weanling hamsters challenged with these tumor lines developed neoplasms. Each of these Ad2-transformed lines contained Ad2 T antigen detectable by complement fixation or immunofluorescence. None of 6 lines contained Ad2 by Sendai fusion with human embryonic kidney (HEK) cells or other contaminating agents by a variety of assays. The difference in oncogenicity in weanling hamsters of Ad2-transformed cells compared to Ad12 - and SV40-transformed cells was not related to the dose of tumor injected or to differences in in vitro growth properties of the cell lines (e.g., doubling times, saturation densities, growth in spinner, or colony formation in soft agar). The tumor inducing capacity of Ad2 newborn tumor lines for newborn and weanling hamsters was not related to the dose of tumor injected. With the two tumor lines tested, weanling rejection of newborn tumor transplants could not be overcome by inocula contain-25 times the usual dose of tumor suspension. These same two newborn tumor lines produced progressively enlarging neoplasms in 17-77% of syngeneic weanling hamsters which had been thymectomized as newborns. The lack of oncogenecity of hamster cells transformed in vitro by Ad2 supported our speculation that the SV40 genome in cells transformed in vivo by the $Ad2^+ND_4$ recombinant could be altering immunological determinants involved in tumor cell rejection.

Viral DNA sequences and gene products in hamster cells transformed by Ad2.

Complementary strand-specific adenovirus DNA of full length or from endonuclease Bam HI fragments was used as a probe to estimate the fractional representation and abundance of viral sequences in five hamster cell lines AD2HEI-5 transformed with UV-inactivated Ad2. The fraction of the viral genome present in the five transformed cell lines varied from 44% in the Ad2HE5 cell line to 84% in the Ad2HE3 cell line. The number of viral DNA copies per diploid cell equivalent ranged from 1.8 in the Ad2HE1 line to 7.1 in the Ad2HE4 line.

In vivo labeling with ^{35}S-methionine followed by immunoprecipitation with an antiserum against Ad2 early proteins revealed viral-specific polypeptides with molecular weights of 42,000 to 58,000 in extracts from all five hamster cell lines. Several other early viral polypeptides were detected in some of the Ad2 transformed hamster cell lines.

Studies are underway to determine the specific regions of the Ad2 genome that are contained in the Ad2HE1 line of Ad2 transformed hamster embryo cells and to determine which of the messenger RNA's produced in these cells are coded for by these Ad2 DNA containing regions. Preliminary results indicate that RNA coded for by the early region on the left hand end of the right strand (the "transforming" region) is present; in addition, RNAs representing regions of the Ad2 genome transcribed late during infection (i.e. after viral DNA replication begins) are also present but appear in patterns not typical of RNA from cells productively infected with Ad2.

Role of the hamster cellular immune response in Ad2 transformed cell oncogenesis

The tumor-inducing capacity of two Ad2 transformed hamster cell-induced lines (Ad2HTL3 and Ad2HTL6) was tested in immunocompromised hosts. An average of 35.2% of neonatally thymectomized, syngeneic, weanling hamsters developed progressively enlarging tumors when injected subcutaneously with tumor suspensions prepared from these lines, while no tumors were observed in normal, syngeneic, weanling hamsters challenged with the same inocula. The susceptibility of neonatally thymectomized hamsters to tumor challenge was directly related to the degree of immunosuppression observed following thymectomy as indicated by the amplitude of the in vitro response of whole blood cultures to concanavalin A. Pretreatment of thymectomized weanlings with syngeneic adult lymphoid cells resulted in a significant reduction in tumor susceptibility ($p = 0.03$). These findings suggest that the maturation of a thymus-dependent cell-mediated immune response in hamsters during the first 21 days of life results in the rejection of Ad2-transformed cells.

Developmental hamster immunobiology related to tumor rejection in the Ad2 transformed hamster cell system

Transplantation of the Ad2HTL newborn tumor line to suckling hamsters 1-15 days old demonstrated that maturation events in the hamsters immune system between 6 and 7 days were responsible for tumor rejection. Very little data has been published concerning the ontogeny of the hamster immune response; however, based on data from other species, it has been predicted that hamster thymus and spleen cells should respond to T cell mitogens within a few days of birth and that suckling hamsters should be able to reject skin allografts at 8 days of age. To begin to evaluate the development of cellular immune responses in hamsters, cells from bloods and spleens from different aged animals were tested for their ability to respond to the mitogen conconavalin A (ConA) and to respond in the mixed lymphocyte reaction. The results of these studies indicate that hamster spleen cells begin to respond to ConA by age 5 days and attained 100 percent of adult type responses by age 15 days. Cells in the blood did not respond to ConA until age 25 days.

Hamster spleen cells did not begin to respond in the mixed lymphocyte reaction until 18 to 25 days. To determine if the lack of ConA responsiveness of hamster spleen cells before age 5 days was due to T cell immaturity or immaturity of another cell component which was required for this response, X-ray inactivated adherent and nonadhereent (to distinguish between lymphocytes and macrophages) adult peritoneal exudate cells were mixed with spleen cells from 3 day old hamsters and the mixtures tested for their ability to respond to ConA. These results showed that, in the presence of populations of adherent peritoneal exudate cells, 3 day old hamster spleen cells readily responded (stimulation indices of 13 to 35) compared to spleen cells alone (stimulation index of 1 to 2) or spleen cells mixed with nonadherent cells (stimulation index of 1 to 3). These results imply that immaturity of cell populations which cooperate with lymphocytes in the response to ConA are most likely responsible for the apparent immaturity of this reaction in hamsters less than 5 days old.

As an in vivo corollary to the evaluation of immune cell responses to mitogen

and foreign antigens, the inflammatory response to tumor development in newborn, weanlings and weanlings thymectomized during the first 24 hours of life was examined histopathologically in animals injected with suspensions of Ad2HTL3 and Ad2HTL6 tumors. In newborn animals multiple tumor nodules developed within 5 days and were quickly enclosed by highly vascular connective tissue. New capillaries were seen entering these nodules. During the first few days inflammatory cells consisted mainly of polymorphonuclear leukocytes with some lymphocytes and histocytes around the forming nodules. In sections with cells invading the surrounding connective tissue, there was no inflammatory response. The response to tumor development in thymectomized weanlings was similar to that observed in newborns. In normal weanlings, tumor nodules developed early but were regressing by 5 days. Some new vessel formation was present during the first few days of tumor nodule development but this was less impressive than the intense vascular response noted in newborns. Inflammatory cells consisted of polymorphonuclear leukocytes during the first day followed by the rapid appearance of an intense lymphohistiocytic infiltrate. After 7 days, the tumor nodules consisted of mostly granulation tissue with many multinucleated giant cells and focal calcification.

Immunogenicity of Ad2 transformed hamster cells and transformed cell-induced tumor lines

To determine the possible role of Ad2 transplantation antigen (TSTA) in the rejection of tumors transplanted to weanling hamsters, several types of studies are underway. An assay has been developed for Ad2 TSTA using an Ad2 tumor line (Ad2HTL1) which had been adapted to grow in adult hamsters. Immunization with Ad2 significantly protected (defined as a resistance index *RI) greater than 10) hamsters against a challenge with Ad2HTL1. Immunization with SV40 did not convey significant protection (RI between 1 to 10) in these experiments. This assay is being used to study the immunogenicity of Ad2 transformed cell lines and newborn and weanling tumor lines developed from these transformed cells. Results thus far indicate that 3 Ad2 transformed cell lines (Ad2HE1, Ad2HE3, Ad2HE6) and the tumor lines established from them (Ad2HTL1, Ad2HTL3, Ad2HTL6) contain a common Ad2 TSTA which during immunization with x-irradiated cells conveys protection to hamsters challenged with Ad2HTL1. Differences in RI did not correlate with the differences in the transplantability of the newborn tumor lines to newborn and weanling hamsters. Adult hamsters that had rejected tumors induced by viable Ad2HTL3 and Ad2HTL6 cells were also protected when challenged with Ad2HTL1. These results show that whether they are accepted or rejected by weanling hamsters, each of these lines contains detectable Ad2 TSTA. Such findings appear to exclude the possibility that certain tumor lines can be transplanted to older animals because they lose detectable Ad2 TSTA. To determine whether a reducation in the concentration of Ad2 TSTA is responsible for the transplantability of certain newborn tumor lines to adults, the amount of immunizing antigen in suspensions of different tumors is being evaluated.

Difference in the transplantability of virus-induced neoplastic cells in in-bred hamsters

Tumor cells behave like normal cells in that they are usually rejected when transplanted as allografts to histoincompatible hosts.Since hamsters are

suspected to lack resistance to transplantable tumors of viral origin, we have been studying the transplantability of our transformed cells and tumor lines to different strains of inbred hamsters. Eleven Ad2, Ad12 and SV40 transformed LSH cell lines and LSH tumor lines established from them are being transplanted to syngenic LSH hamsters, CB hamsters which are histocompatibility complex (MHC) disparate, and PD4 hamsters which are MHC identical but disparate at minor H loci. Initial findings show that Ad2 transformed cells produced tumors only in newborn LSH hamsters. Ad2 tumor lines (Ad2HTL1, Ad2HTL3) adopted to grow in LSH adults by serial passage in neonatal hamsters produced tumors readily (TPD_{50} $10^{2.3}$ to $10^{4.97}$ 0.2 ml of tumor suspension) when transplanted to LSH adults. These same lines produced tumors inefficiently (TPD_{50} $10^{0.0}$ to $10^{2.2}$/0.2 ml) when transplanted to adult CB hamsters and of intermediate efficiency (TPP_{50} $10^{1.4}$ to $10^{3.7}$/0.2 ml) when transplanted to PD_4 hamsters. When 10^6 to 10^7 Ad12 transformed LSH cells were inoculated into adult LSH hamsters 84% developed tumors. When the same cell doses were injected into CB hamsters only 33% developed tumors. Tumor lines established in LSH hamsters from Ad12 transformed cells produced tumors in a pattern similar to the Ad2 tumor lines when transplanted to adult LSH, CB, and PD_4 hamsters. When 10^6 and 10^7 SV40 transformed LSH cells were injected into adult LSH and CB hamsters, 100% and 97% respectively developed tumors. SV40 tumor lines produced tumors with equal efficiency in all 3 hamster strains. Thus, our Ad2 induced neoplastic LSH cells produced tumors only in immunoimmature syngeneic LSH hamsters; our Ad12 induced neoplastic LSH cells produced tumors efficiently in both immunoimmature and immunomature syngeneic LSH hamsters but produced tumors less efficiently in allogeneic hamsters; our SV40 induced neoplastic LSH cells produced tumors efficiently in both syngeneic and allogeneic hamsters.

Current ideas about the role of TSTA and allograft rejection of viral induced neoplasms do not account for the finding above. In this regard, we are considering the possibility that the differences in the transplantability of these various cell lines are a reflection of the unrecognized manner in which tumor and allograft determinants on the surface of hamster cells are altered during transformation events by a particular viral agent. Studies are underway to further clarify these findings.

Significance to Biomedical Research

The results of our recent studies impinge on tumor immunology and the possible role of antigenic determinants on cell surfaces in determining the outcome of viral-induced malignancy. The availability of well-characterized tumor lines, which are transplantable to both immunoimmature and immunomature hamsters and tumor lines, which are transplantable to immunoimmature but are consistently rejected by immunomature animals provides a useful system for identifying and studying the maturation of the functions of the hamster cellular immune system responsible for the rejection of viral-induced tumors. The central theme of tumor virology is the concept that viruses induce the malignant state (i.e., the ability to produce tumors in a susceptible host) in cells by a process called transformation. A number of studies (including our own work with the nondefective Ad2-SV40 hybrids) with different agents have associated transformation and tumor induction with the functioning of a specific region of the viral genome and in some cases, with a specific gene product. Paridoxically,

many virus-transformed cells which possess the properties ascribed to the transformed state fail to produce tumors when injected into either syngeneic immunomature or immunoimmature hosts. The reasons for this lack of transformed cell oncogenicity for a host that should be susceptible are poorly understood. Our studies with viral transformed hamster cells provide data which pertain to this question. At this juncture, we interpret our results with the Ad2 and SV40 transformed LSH hamster cell system as suggesting that, during the process of transformation, antigenic determinants on cell surfaces in addition to TSTA are altered. Some of these determinants are associated with graft rejection by allogeneic hosts; others are associated with the recognition and rejection of tumor cells by syngeneic hosts. An understanding of these cell surface modulations and the role of the viral genome in the alteration of their function would be a significant step in understanding the mechanism by which viruses convert normal cells to malignant ones.

Publications

Patch, C. T., Levine, A.S., and Lewis, A.M., Jr.: The adenovirus-SV40 hybrid viruses. Comprehensive Virology, 13: 495-542, 1979.

Johansson, K., Persson, H., Lewis, A.M., Jr., Petterson, U., Tibbetts, C. and Philipson, L.: Viral DNA sequences and gene products in hamster cells transformed by adenovirus type 2. J. of Virol., 27: 628-639, 1978.

Cook, J.L. and Lewis, A. M., Jr.: Host response to adenovirus 2 - transformed hamster embryo cells. Cancer Res., 39: 1455-1461, 1979.

Lewis, A.M., Jr. and Martin, R. G.: The oncogenicity of simian virus 40 deletion mutants that induce altered 17k t-proteins. Proc. Nat. Acad. of Sci., in press.

Patch, C.T., Hauser, J., Lewis, A. M., Jr., and Levine, A.S.: A method for determining the extent and copy number of overlapping and non-overlapping segments of integrated viral genomes. J. of Virol., in press.

Cook, J.L. and Lewis, A.M., Jr.: Age-related and thymus-dependent rejection of adenovirus 2 - transformed cell tumors in the Syrian hamster. Cancer Res., in press.

Lewis, A. M., Jr. and Cook, J.L.: The association of tumor induction by ultraviolet light inactivated adenovirus 2-SV40 recombinants with a specific segment of SV40 DNA. J. of Nat. Cancer Inst., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AI-00018-13 OSD

PERIOD COVERED

TITLE OF PROJECT (30 characters or less)

Biological and Biochemical Characterization of Human Papovaviruses.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Kenneth K. Takemoto	OSD, NIAID
OTHER:	Sheila Bond	OSD, NIAID
	Tatsuo Miyamura, Visiting Fellow	OSD, NIAID

COOPERATING UNITS (if any)

Peter Howley, Ming Fan Law, LP, NCI; George C. Fareed, UCLA, Los Angeles, CA; Hawley Linke, UCLA, Los Angeles, CA., L.W. Law, NCI.

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SECTION

Cellular Virology Section

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The principal goal of this unit continues to be the detailed biological and biochemical characterization of the human papovaviruses, BKV and JCV. During the past six years, most of the research efforts of this unit were directed toward studies with BKV since this virus could easily be grown in cell cultures of various types. JCV, on the other hand, has been difficult to propagate in vitro, and therefore has remained a relatively unknown virus. During the past year, however, a major advance was made when we were able to grow JCV in two different readily available cells, human amnion and human embryonic kidney cells. This will now enable us to conduct detailed investigations on the biology of this important virus.

Studies are continuing on the mechanism of persistent infection of human fetal brain cells by BKV. It has been established that viral genomes exist in episomal form in these cells, and that rare cells in the culture spontaneously undergo lytic cycles to produce infectious progeny. This type of virus-cell interaction may explain how these papovaviruses persist indefinitely in the human host.

PHS-6040
(Rev. 10-76)

PROJECT DESCRIPTION

Persistent BK virus infection. We reported last year on the establishment of a unique, heretofore unrecognized type of persistent viral infection in cell culture. In this system, BK virus was shown to persist indefinitely in transformed human fetal brain cells. The mechanism whereby virus persists in the cells has been determined. It was found that all cloned lines contained viral DNA in non-integrated, episomal form. By restriction endonuclease analysis, the episomal viral DNA was shown to be identical to parental DNA and was infectious. The number of episomes per cell was between 5 to 10. It is postulated that in an occasional cell, the episomal DNA is spontaneously "induced" to go into a complete replicative cycle and viral progeny is produced. The persistent state is thus perpetuated, even in the presence of potent antiserum. We speculate that this type of virus-cell interaction can perhaps explain how these viruses persist in their natural hosts.

Another unusual feature of these cells is that they are transformed and have most of the properties of transformed cells. They grow in low serum medium, form colonies in soft agar, are immortal, and produce tumors in nude mice. However, unlike the usual papovavirus transformed cells, they do not contain T-antigens detectable by immunofluorescence. By immunoprecipitation and analysis in gels, there appears to be an abberant T-protein produced with a molecular size in the range of 40K. Whether this protein is of viral or cellular origin is being determined.

Replication of JC virus in non-fetal human cells. JC virus (JCV) has now been shown to be the causative agent in progressive multifocal leukoencephalopathy (PML), having been isolated from or identified in over 30 cases of the disease. Studies on this important virus have been severely limited due mainly to the difficulty in growing the virus; until recently, it has been grown only in human fetal glial cells, which are difficult to obtain. In the past year, we have successfully adapted JCV to grow in 2 types of commercially available cells, human embryonic kidney and human amnion cells. Both kinds of cells support JCV replication with full yields of virus. Serological and biochemical analysis of the adapted JCV showed that the virus was identical to the original strain grown in human fetal glial cells. Detailed biological and biochemical investigations on this virus can now be performed and a more complete characterization of JCV can be expected within the next few years.

BKV helper function for adenovirus replication. Previous comparative studies on common early viral functions between the simian and human papovaviruses, SV40 and BKV, have established a relatedness between the 2 viruses. Thus, they share immunologically related T-antigens and BKV can complement the growth of temperature sensitive SV40 mutants which are defective in early gene functions. To understand more fully the functional similarities between BKV and SV40, the helper function for adenovirus growth by BKV in non-permissive monkey cells was studied. During the course of these experiments, it was observed that replication of BKV in CV-1 monkey cells was primarily abortive at 37°C. However, when infected cells were incubated at high temperature (40°C) the cells became permissive for BKV replication and high virus yields were obtained.

Based on this finding, experiments to determine helper function for adenovirus growth in monkey cells by BKV were performed at abortive (37°C) and permissive (40°C) temperatures. Results of these experiments showed that while enhancement of adenovirus replication occurred in cells co-infected with BKV and incubated at 37°C, there was a 10-fold greater degree of enhancement at 40°C. This was probably due to an increased production of T-antigen at the higher temperature. These experiments thus provide additional information on the high degree of relatedness between the simian and human papovaviruses and is in agreement with the recent findings of extensive homology between the genomes of the 2 viruses obtained by DNA sequencing studies.

Common sequences in the genomes of JC, BK, and SV40. In studies done in collaboration with Drs. Law and Howley (NCI), the DNAs of the primate papovaviruses SV40, BK, and JC were analyzed for nucleotide sequence homology. Under non-stringent conditions, extensive homology was found throughout the genomes of the 3 viruses, which correlate well with the previous observations that the structural (viral) as well as non-structural (t-proteins) antigens of the primate papovavirus are immunologically related. The region of strongest homology among the 3 genomes was localized in the late region between 0.76 to 0.85 map units coding for VP2.

Significance to Biomedical Research. The human papovaviruses are ubiquitous viruses which are world-wide in distribution, infecting children at an early age and persisting thereafter throughout life. One of them, JCV, has clearly been determined to be the causative agent of PML. The papovaviruses represent a class of persistent viruses whose possible role in chronic diseases, including cancer, need to be determined. They have been demonstrated to be oncogenic in tissue culture and in animals, thereby providing important models for the study of viral oncogenesis.

Proposed course. The ability to propagate JC virus in non-fetal cells will now enable us to conduct detailed investigations on the biology and biochemistry of this virus which were heretofore not possible. An immediate objective is to analyze the DNA of JCV virions since previous data indicated extensive heterogeneity which probably accounted for the poor growth and low yields of virus. The system of persistent BKV infection in T-antigen negative, transformed human fetal brain cells will continue to be investigated.

Publications

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Bond, S.B., Howley, P.M. and Takemoto, K.K. Characterization of K virus and its comparison with polyoma virus. J. Virology 28, 337-343, 1978.

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Takemoto, K.K., Howley, P.M. and Miyamura, T. JC human papovavirus replication in human amnion cells. J. Virology 30, 384-389, 1979.

Law, M.F., Martin, J.D., Takemoto, K.K. and Howley, P.M. The co-linear alignment of the genomes of papovaviruses JC, BK, and SV40. Virology (In Press)

Miyamura, T. and Takemoto, K.K. Helper function for adenovirus replication in monkey cells by BK human papovavirus. Virology (In Press).

Awards and Honors

Invited lecturer, Virology '79 Lecture Series, The Institute for Medical Research, Camden, New Jersey, January 4, 1979. "The Papovavirus Group."

American Society of Microbiologists Symposium on Viruses and Human Cancer, May, 1979, Los Angeles, California. "Human Papovaviruses: Search for evidence of possible involvement in human cancer."

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-A1-00019-05 OSD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Studies on the Treatment of Disease with the Interferon System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Hilton B. Levy	Biochemist	OSD, NIAID
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COOPERATING UNITS: Dr. Arthur Levine, NCI; Dr. King Engel, NIANDS; Dr. John Hooks, NIDR; Maj. Edward Stephen, USAMRIID; Dr. Chester Liu, USAMRIID; Maj. Don Harrington, USAMRIID; Dr. Martin Lerner, Wayne State Univ.; Dr. Larry Crane; Wayne State Univ; Drs. Herbert Oettgen and Susan Krown, Sloan Kettering Inst.;

COOPERATING UNITS (if any) Dr. Beatrice Lampkin, Children's Hospital Center, Cincinnati, Ohio; Dr. Mosmorine, Connaught Laboratories, Canada; Dr. Goodwin Hilfenhaus, Behringeverke, Germany; Dr. Tagir Bektemirov, Gamalaya Institute, USSR; Dr. Edward Lvosky, Litton Bionetics.

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The nuclease-resistant, primate-effective interferon inducer, poly inosinic polycytidylic acid, poly lysine, carboxymethyl cellulose (Poly ICLC) has a variety of physiological activities. In addition to inducing interferon in primates, it is an immune adjuvant and a radioprotective agent. It is currently being evaluated in 3 collaborative clinical studies, and 3 more are being developed. It appears to have had ameliorative effects in 4 patients; one with lymphoblastic leukemia, one cancer patient whose widely disseminated Herpes lesions disappeared after treatment, and 2 patients with chronic relapsing polyneuropathy. A drug without carboxymethycellulose has been prepared and is being evaluated.

PHS-6040
(Rev. 10-76)

Project Description

This past year has been one of preparing to do larger scale testing of poly ICLC in man. This has included actual clinical studies and studies of a more basic type.

Programs are ongoing with the Sloan Kettering Institute, where a phase I study of the drug in terminal cancer patients is being done. They are using a different dosage and administration schedule than that which we used with Dr. Levine of the NCI. All that can be said at this writing is that the patients are making good levels of interferon. One leukemia patient had widely disseminated herpes continuously for 1 and 1/4 years. One week after going onto the drug, the herpes lesions disappeared and have not reappeared.

A phase II study has been initiated with the Children's Cancer Study Group, a multi-institution collaborative organization. The present protocol is designed to treat acute lymphoblastic leukemia at stages earlier than terminal. No data are available as yet.

The Policy Network of NCI accepted poly ICLC as a high priority drug, and is undertaking its manufacture, packaging and distribution. They also are beginning a long-range toxicology study, the results of which will be available for NIAID use in treating patients with viral disease. We have developed a protocol together with NCI to treat a variety of solid tumors and leukemias. The protocol has passed NCI, Clinical Center and NIAID review. There are extensive immunological studies described in this protocol.

There have been written the following protocols for clinical studies which are almost ready to submit for NIAID approval.

1) With Drs. Alexanian and Gutterman of M.D. Anderson Hospital - to treat patients with multiple myeloma in comparison with their study using exogenous interferon.

2) With Dr. Brian Durie of University of Arizona - to treat multiple myeloma and to study the effect of the drug on a variety of immunological parameters.

3) With Drs. Mosely and Rakela of University of Southern California Medical School liver unit - to treat patients with hepatocellular carcinoma associated with chronic hepatitis B infection.

Poly ICLC, like the parent poly I Poly C, is pleiotropic. In addition to its ability to induce interferon, it has demonstrated the following action: To date, it has been shown to be even at low doses, an effective adjuvant with the following weak vaccines; Venezuelan Equine encephalitis, Japanese B encephalitis, Swine Flu, hemophilus influenzae polysaccharide, Rift Valley fever and Herpes envelope antigens. With strong antigens, such as albumin and pneumococcal polysaccharide type III, if anything, there is an inhibitory action on antibody production. Monkeys receiving poly ICLC showed a marked, but transient increase in the number of small lymphocytes in the paracortex of the nodes draining the site of injection, as well as an increase in migration rate of lymphocytes from high endothelial venules.

Poly ICLC, like a number of other interferon inducers is a radio protective agent. Mice given Poly ICLC can tolerate a significantly increased amount of X-irradiation. While the mechanism of protection is not clear, it is probably associated with a strong stimulation of marrow stem cells as shown by an increase in endogenous colonies in the spleen.

In a study with Dr. Engel of NIAMDS a year and a half ago, a patient with chronic relapsing polyneuropathy was started on poly ICLC. After six weeks of treatment the patient went from a state of almost complete paralysis to one in which he was able to walk six miles, and lift weights over his head. He continues to be well, but requires weekly or biweekly injections. Otherwise, weakness begins to set in again.

Another patient with a similar illness also made a dramatic recovery but doesn't appear to require continued treatment. Immunologic studies are being done on these patients.

Connaught Laboratories, the manufacturer of Salk type polio vaccine, has had a serious problem in obtaining enough monkey kidney tissue culture to grow the polio virus. The monkeys are chronic carriers of foamy virus, and there is barely time to get a harvest of poliovirus from the primary tissue culture growth, before the foamy virus destroys the tissue culture. They could increase the amount of tissue culture by 10 fold if they could make secondary cultures. In two collaborative experiments they found that by treating the monkeys with poly ICLC before removing the kidneys, they could reduce the virus titer sufficiently so that secondary cultures could be made. They plan to make the poly ICLC themselves and try it again. If successful, they will consider applying it to their production schedule.

Commercial interest in poly ICLC has been expressed by Pasteur Development Corporation in France, Ciba-Geigy in Switzerland and Merck, Sharpe and Dohme in this country.

There has been some concern over the presence of carboxymethylcellulose (CMC) in poly ICLC. While CMC has been used in parenteral medicines for years, the fact is that there is no known enzymatic pathway for its degradation. Monkeys and humans who have received poly ICLC over two years ago have shown no adverse reactions, nor have those who received CMC as a vehicle for steroid administration. However, because it would be easier to formulate and might allow for the preparation of a more concentrated solution, we have tried to prepare a poly I poly C poly lysine complex without CMC. By taking advantage of the known effects of ionic strength and temperature on the components, we have succeeded in preparing a series of such complexes that are resistant to hydrolysis by RNase, and which induce interferon in monkeys and mice. Much work needs to be done before we can give this poly ICLC to people.

We have studied the effect of changing the size of the poly lysine and that of the poly I and poly C on Tm, nuclease resistance and capacity to induce interferon in monkeys. Poly lysine of molecular weight 2000 forms an ineffective complex, poly lysine of molecular weight 27,000 is close to maximally effective. 9s Poly I and Poly C form a much more effective complex than does smaller poly I or poly C. Our standard poly ICLC is made with 9s Poly I, 9s Poly C, 27,000

mol. wt. poly lysine and high viscosity CMC.

Future Plans:

1) It is hoped in the next year to expand clinical work with patients with malignancies, emphasizing the following questions: a) Are there some tumors that are responsive? b) What are the effects on virus diseases in these patients? c) What are the effects of the drug on the immune system in man?

2) With NCI it is planned to do long-term toxicological studies in mice and monkeys.

3) Through the use of the contract mechanism we plan to examine the question of stability of the drug, as well as some biochemical and immunological matters that bear on therapy.

4) In a collaborative study with Dr. Liu of USAMRIID we plan to examine the effect of poly ICLC and interferon on a large variety of cardiovascular functions in monkeys.

Publications

Harrington, D. G., Crabbs, C. L., Hilmas, D. E., Brown, Jr. R., Higbee, S.A., Cole, . E., Levy, H.B.: Adjuvant effects of low doses of a nuclease-resistant derivative of polyinosmic acid on antibody responses of monkeys to inactivated Venezuelan equine encephalomyelitis virus vaccine. Infection and Immunity, Apr. 1979, p. 160-166.

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Hilmas, D. E., Stephens, E. L., Spertzel, R. O., Levy, H. B. Use of PICLC for the prophylaxis and treatment of Venezuelan equine encephalomyelitis virus infection in nonhuman primates. Current Chemotherapy, 1978.

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Levy, H. B., Lvovsky, E. Tropical treatment of vaccinia virus infection with an interferon inducer in rabbits. Journal of Infectious Diseases, Vol. 137, No. 1. January, 1978.

Levy, H. B., Hilmas, D. E. Evaluation of a nuclease-resistant derivative of PLCLC as a radioprotective agent. Radiation Research, 77, 1979.

Stephens, E. L., Hilmas, E. E., Levy, H. B., Spertzel, R. D. Protective and toxic effects of a nuclease-resistant derivative of PIC on Venezuelan equine encephalomyelitis virus in Rhesus monkeys. Journal Infectious Diseases, Vol. 139 No.3, 1979.

Lerner, M. E., Levy, H. B. Physiological accompaniments of sustained interferonemia induced in man by poly ICLC. Inf. Immun. Accepted.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-A1-00131-12 OSD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Mechanisms of Hypersensitivity in Histocompatible Inbred Guinea Pigs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Dr. Sanford H. Stone Chief, Immunology Section OSD, NIAID

COOPERATING UNITS (if any) Dr. Cedric S. Raine, Division of Neuropathology, Albert Einstein College of Medicine, New York, N. Y.; Dr. Richard H. Quarles, D.M.N., NINCDS, Bethesda, MD.; Dr. Robert B. Nussenblatt, CB, NEI, Bethesda, MD.

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2	1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

For some years, we have been using the unique tool of inbred histocompatible guinea pigs for investigation of mechanisms of hypersensitivity in the pathogenesis of and the protection against infectious and autoimmune diseases. Advantages lie in the ability to perform viable adoptive transfer of lymphoid cells, and in the capacity to study genetic factors in certain hypersensitivity and autoimmune phenomena. We have concentrated recently on the pathogenesis of an age-dependent induction of cataracts in immature guinea pigs undergoing the paralysis and atrophy of autoimmune encephalomyelitis and on the prevention and treatment of this condition. The cataracts are reminiscent of some clinical or experimental entities shown to be due to nutritional deficiency or enzymatic alteration and may be secondary to the autoimmune reaction in the host.

PHS-6040
(Rev. 10-76)

Project Description

Subproject I - Cataract formation in allergic encephalomyelitis of guinea pigs

An unusual pathological event occurred in immature guinea pigs sensitized to spinal cord antigens either actively or passively by lymph node-cell transfer. A significant number of these developed cataracts bilaterally during a severe acute attack of allergic encephalomyelitis (EAE). The lens histology was significant for posterior migration of the epithelium with some eyes showing vacuolization under the capsule. The vitreous, choroid and retina did not show foci of inflammation and the intra-ocular and intracleral portion of the optic nerve showed no evidence of cell drop-out nor round cell infiltration. Histological preparations from the central nervous system (CNS) of the guinea pigs showed the classic picture of acute EAE.

The age-dependency of this eye lesion was quite evident. Both weanlings and newborns, but no adults, developed the opacities; the cataracts were not strain-specific, occurring in both strain 13 and Hartley guinea pigs. The evidence points to the probability that these cataracts are not the direct result of an immunological response, but secondary to the acute paralytic syndrome of EAE.

Subproject II - Protection against chronic EAE by injection of myelin basic protein in incomplete Freund's adjuvant

CNS lesion morphology was examined in detail in inbred strain 13 guinea pigs sensitized for chronic EAE in which the disease was either allowed to develop or was suppressed by injections of myelin basic protein (MBP). Pathologic changes correlated well with the clinical picture; in chronic animals clinical disease was accompanied by inflammation in the CNS including fibrosis and remyelination. Relapses showed the CNS to contain recent changes superimposed upon old lesions. In animals in which the disease was suppressed, clinical signs did not develop, but some early sub-clinical changes were seen morphologically. These lesions were remyelinated promptly and there was no progression in lesion formation. This contrasted with the progression in untreated chronic animals; long-standing disease was characterized by large, burnt-out plaques, with Schwann cell invasion and peripheral nervous system myelination, glial bridges between sub-pial astrocytes and the leptomeninges, and fenestrated blood vessels.

Therapy of established chronic disease (as distinguished from suppression experiments) is under investigation currently using MBP in paralyzed strain 13 guinea pigs. This will require an elaborate set of controls and will be cautiously pursued in view of its relevance to the treatment of multiple sclerosis.

Publications

Raine, C. S., Traugott, U. and Stone, S. H.: Chronic relapsing experimental allergic encephalomyelitis: CNS plaque development in unsuppressed and suppressed animals. Acta Neuropathol. 43:43-53, 1978.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-A1-00190-01-OSD

PERIOD COVERED

TITLE OF PROJECT (80 characters or less)

The Molecular Genetics of Eukaryotic Cells and Their Viruses.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Dean Hamer, Staff Associate	OSD, NIAID
	Mark Israel, Research Associate	OSD, NIAID
	Yoshiaki Ito, Visiting Scientist	OSD, NIAID
	Roy Repaske, Microbiologist	OSD, NIAID
	Kamal Chowdhury, Visiting Fellow	OSD, NIAID

COOPERATING UNITS (if any)

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SUMMARY OF WORK (200 words or less - underline keywords)

The principal goal of this unit continues to be the biochemical characterization of eukaryotic and animal viral genes. Attention has been focussed on the segments of DNA and RNA Tumor virus genomes responsible for the establishment and maintenance of the transformed state. In this regard we have shown that the large form of polyoma tumor antigen plays no role in tumorigenesis mediated by polyoma DNA. SV40 DNA recombinants have been used to elucidate the mechanisms regulating the synthesis and processing of eukaryotic RNA.

The first phase of risk assessment experiments conducted in the P4 facility at Ft. Detrick, Maryland, was recently completed. These studies showed that polyoma virus DNA is not transferred out of E. coli K-12 following the inoculation of susceptible newborn animals with bacteria containing recombinant plasmids or phage.

PHS-6040
(Rev. 10-76)

Project Description:

The DNA Recombinant Research Unit, established in the Summer of 1978, has continued to utilize recombinant DNA procedures to investigate the regulation and structural organization of eukaryotic cells and their viruses. While the main thrust of research activities continues to be the study of the transforming region of SV40 and polyoma virus, new projects which involve the molecular cloning and biochemical characterization of murine retraviruses and the use of the SV40 vector system to evaluate transcriptional regulation of eukaryotic genes has been initiated. Experiments evaluating potential risks associated with recombinant DNA research have also been carried out at the P4 facility located at Ft. Detrick and P2 laboratories on the NIH reservation.

During the year Dr. Daniel Simmons resigned to take a position as Assistant Professor of Biology, University of Delaware. Dr. Dean Hamer joined the Unit last fall following post doctoral training in Dr. Phil Leder's laboratory and will use SV40 recombinants to investigate transcription of mammalian genes. Dr. Yoshiaki Ito joined the group in August following a six-year tenure at the Imperial Cancer Research Fund Laboratories, London and will continue to characterize transforming polypeptides specified by papovavirus genomes. Dr. Roy Repaske became an active participant in the risk assessment experiments coordinated by Drs. Martin and Rowe and perfected the in vitro packaging procedure utilized in "shotgun" cloning. He has recently mastered nucleotide sequencing procedures and will use them to characterize specific segments of cloned DNA.

Research Accomplishments

I. The Oncogenic Potential of Papovaviruses

During the past two years the polyoma (PY) virus system has been used to evaluate the molecular events associated with transformation and tumorigenesis. The PY virus system has at least two advantages over SV40: 1) tumors are readily and rapidly induced in newborn hamsters; 2) the PY viral genome encodes three peptides which play a role in the oncogenic process whereas SV40 DNA contains the genetic information for only two of these three. We have extended our previous studies involving tumorigenesis in newborn hamsters by evaluating the biological activity of viral DNA preparations previously digested with restriction enzymes. These experiments clearly indicate that tumorigenicity of PY DNA is enhanced when the distal portion of the early gene region is interrupted. This was an unexpected result since in the more extensively studied SV40 system, the entire early region must be intact for the initiation and maintenance of the transformed state. Using immunoprecipitation techniques, we demonstrated that tumor cell lines, originally induced by PY virions or PY DNA, contained the small and middle PY tumor (T) antigens (Ags) but not the large form of viral T Ag. Fifteen independent cloned tumor cell lines derived from hamster tumors induced by PY virions or PY DNA were analyzed by blot-hybridization and shown to contain viral DNA sequences specifying the small and middle PY T Ags. PY DNA sequences encoding the large T Ag were invariably interrupted. These data imply that PY large T Ag is not required for the maintenance of the tumor cell phenotype. Studies are currently in progress

to evaluate whether PY large T Ag plays any role in virus mediated tumorigenesis. An effort is also being made to clone the PY DNA sequences integrated into the tumor cell genome. (Israel, Chowdhury, Martin.)

II. The Molecular Structure of Murine Retraviruses

Our unit has initiated a major collaborative effort with Dr. Wallace Rowe's group (LVD) to clone and characterize a variety of murine retraviruses. This research activity grew out of an ealier joint project with Dr. Edward Scolnick's group (Laboratory of Tumor Virus Genetics, NCI) in which Friend Leukemia (FLV), and Harvey Sarcoma (HaSV) viruses were cloned in derivatives of E. coli K12 using a lambdaphage vector. After refining in vitro packaging procedures to permit the "shotgun" cloning of unintegrated viral DNA, large quantities of FLV and HaSV DNAs were prepared and extensively characterized. We concentrated our energies of the restriction mapping of HaSV DNA and showed that one of the six recombinant phage we constructed contained a triplication of viral DNA sequences that flank both ends of the integrated HaSV DNA. Work is continuing on the stability of the reiterated HaSV DNA sequences in both eukaryotic and prokaryotic host cells and determining the fine structure of the reiterated DNA segment. (Chan, Repaske, Martin.)

More recently we have begun the molecular cloning of the endogenous retraviral DNA sequences present in the AKR mouse. Thus far, we have obtained recombinant phage preparations containing integrated: 1) ecotropic AKV; 2) xenotropic; and 3) two MCF DNA sequences. The cloned AKV and xenotropic preparations appear to represent complete viral genomes. These retraviral DNA inserts will be biochemically characterized and subgenomic viral DNA segments will be purified for use as a probe in hybridization experiments for the detection of specific regions of viral DNA. (Chan, Repaske, Martin.)

III Regulation of Eukaryotic Genes

In order to provide a biological assay for regulatory sequences present in eukaryotic DNA as well as mutants derived from them, we have developed SV40 host vector systems that allow us to introduce new genetic information into animal cells. During the past year, we have constructed recombinant molecules containing chromosomal mouse globin genes linked to an SV40 vector and infected monkey cells. We find that the mouse globin gene signals for transcription, processing and translation are recognized in monkey cells resulting in the production of substantial quantities of globin. Further, by preparing mutant forms of SV40, we have characterized discontinuous eukaryotic genes and the splicing of mammalian RNA. Specifically, the minimum size of the recognition site, the species, cell type, and distribution of the splicing enzymes, and the physiological role of splicing in stable RNA formation has been investigated.

We are now attempting to extend this system to the human globins and are particularly interested in using SV40 recombinants to elucidate the molecular lesions in various hemoglobinopathies. We have also initiated a project to form SV40 recombinants carrying hepatitis virus type B sequences. Such hybrids could be very useful in mapping the hepatitis genome and, eventually, for preparing diagnostic and therapeutic reagents. (Hamer)

IV. Assessment of Risks Associated with Recombinant DNA Research

In December 1975, Drs. Malcolm Martin and Wallace Rowe were asked by the Recombinant DNA Advisory Committee to conduct experiments in NIH containment facilities to evaluate potential risks associated with recombinant DNA experimentation. The first phase of such studies was carried out in P4 containment facilities located in Frederick, Maryland or on the NIH reservation and involved the inoculation of weanling mice or newborn hamsters with E. coli K12 containing polyoma-plasmid or polyoma-lambdaphage recombinants. In both animal systems, the polyoma-recombinants were not transferred out of their E. coli hosts following parenteral inoculation. As controls, polyoma DNA, liberated from the prokaryotic vector DNA, was infectious (mice) or tumorigenic (hamsters). Mice inoculated with purified recombinant DNA preparations containing a single copy of viral DNA never developed a virus infection whereas, a few animals infected with a recombinant phage DNA preparation harboring a head-to-tail dimer of viral DNA developed an infection. Intact recombinant DNA preparations as well as purified phage particles induced tumors in 5-19% of inoculated animals, a value similar to that observed following injection of supercoiled PY DNA (19%). It should be emphasized that potentially infectious or tumorigenic recombinant DNA molecules are not transferred out of EK2 hosts into mammalian cells in either of the animal model systems we used. Even in those cases in which animals inoculated with recombinant phage or purified recombinant DNA did develop a viral infection or tumors in worst case experiments conducted in a laboratory setting, the biological activity of recombinant preparations was at least a million-fold less active than polyoma virus particles.

We have recently received polyoma plasmid and polyoma lambda recombinants in EK1 E. coli host cells, which were constructed by investigators in Europe. We have agreed to begin animal testing studies in the fall of 1979. In addition, we plan to inoculate bacteria containing cloned Harvey Sarcoma and Friend Leukemia virus DNAs into appropriate test animals to extend our risk-assessment analysis to another virus (retravirus) system. (Drs. Martin and Rowe and their friends.)

Publications

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Howley, P.M., Israel, M.A., Law, M.F., and Martin, M.A.: A rapid method for detecting and mapping homology between heterologous DNAs. Evaluation of polyomavirus genomes. Journal of Biological Chemistry, 254:4884-4891, 1979.

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Simmons, D.T., Takemoto, K.K., and Martin, M.A.: Properties of simian virus 40 and BK virus tumor antigens from productively infected and transformed cells. Virology 85:137-145, 1978.

Simmons, D.T., Chang, C., and Martin, M.A.: Multiple forms of polyoma virus tumor antigens from infected and transformed cells. J. Virol. 29:881-887, 1979.

Honors and Awards

Dr. Malcolm Martin was awarded the Public Health Service Superior Service Award in May for his activities involving recombinant DNA research. Dr. Martin continues to serve on the Editorial Boards of Journal of Virology and Journal of Biological Chemistry. In June, he completed a four year appointment as a member of the Virology Study Section, DRG, NIH. In April, Dr. Martin was invited to deliver a lecture at the Cogene-The Royal Society of London Meeting on Recombinant DNA held at Wye College, Kent, England.

LABORATORY OF BIOLOGY OF VIRUSES
1979 Annual Report
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Annual Report of the Laboratory of Biology of Viruses
National Institute of Allergy and Infectious Diseases, NIH
October 1, 1978 to September 30, 1979

The goal of the Laboratory of Biology of Viruses is to determine how viruses replicate. At the molecular level, this involves investigation of the structure of the virion and of its purified component parts, of mechanisms of replication and transcription and expression of the viral genome. Emphasis is also placed on cell-virus interactions including the process of cell transformation. We believe that information of this kind, although difficult to obtain, is fundamental to understanding disease processes and vital to achieving a rational basis for chemotherapy.

Papovaviruses

The current studies have tried to define some of the processes responsible for the formation of viral messenger RNA molecules. Thus far, these studies have provided the precise structures of the viral mRNA molecules, have defined the nature of the templates used for the synthesis of viral transcripts, have located promoters on the SV40 genome that are recognized by E. coli RNA polymerase and have compared SV40 DNA I and SV40 DNA I nucleoprotein complexes as templates for transcription.

The Structure of Early and Late Viral mRNA

The mechanism of splicing of the early and late cytoplasmic species of SV40 mRNA has been studied using a technique which first involves the isolation of specific regions of the viral genome. Restriction enzyme cleavage of SV40 DNA generates specific DNA fragments which can be fractionated using column chromatography. These fragments are then annealed to viral mRNA and the reaction products are fractionated by velocity sedimentation. The DNA fragment which is annealed to the viral mRNA is used as a primer for the enzyme, reverse transcriptase, and a complementary DNA copy of the mRNA is made. The sequence and structure of the cDNA has been compared with the known nucleotide sequence of SV40. This procedure has been used to characterize the late mRNA which codes for the major structural viral coat protein and has provided direct physical evidence for two early mRNA species. The regions of the DNA that have deleted in both species of early mRNAs have been determined. Also, the nucleotide sequences across these spliced regions have been determined and correlated with the mRNA coding for the large T and small t antigens. These two mRNA species were determined to have the same 5' termini which would suggest two levels for control of genetic expression. One would be the regulation of initiation of transcription at a common promoter; the other would involve post-transcriptional splicing.

To further characterize the mechanism of transcription of early SV40 RNA, nascent RNA chains that are attached to template DNA molecules are being isolated and characterized by the same methodology employed to study the processed cytoplasmic species of early viral RNA. Transcription

complexes have been isolated from infected cells which continue viral mRNA synthesized *in vitro*. The structure of these nascent chains gives insight into the initial transcription products and the mechanisms that operate to regulate transcription. We are investigating differences between RNA's formed *in vivo* and *in vitro*. The structure of nascent chains will provide precise information about the primary products of transcription. We are also studying the use of isolated nuclei for the study of the regulation of transcription. Isolated nuclei are able to sustain *in vitro* synthesis of viral RNA. In this case, the SV40 template in the cell nucleus is present as a nucleoprotein complex containing the five histones. This is in contrast to *in vitro* synthesis of nascent RNA chains described above which uses a less well defined transcriptional complex.

These nascent RNA molecules will be probed with specific SV40 DNA fragments to determine their structure. In order to acquire a library of these specific fragments, recombinant technology has been employed. Cloning of SV40 DNA fragments using the plasmid pBR322 and E. coli should provide sufficient amounts of specific DNA fragments which can be used to probe nascent RNA molecules and determine the mechanisms operating during regulation of transcription.

Since sufficient amounts of specific SV40 DNA fragments can be obtained from cloning, we will be able to study how synthesis of specific RNA sequences are affected by mutations within the genome. Further characterization of RNA species obtained following Proflavin treatment, which blocks RNA processing, will aid our understanding of the mechanism that operated to regulate transcription. (Thompson, Bina-Stein, Salzman)

Localization of RNA Polymerase Promoters on SV40 DNA

Transcripts of SV40 DNA synthesized by *Escherichia coli* RNA polymerase have been characterized. This model system has been used for the development of new methods applicable to the analysis of the mechanisms involved in the synthesis of the viral messenger RNAs in SV40-infected cells. It has been previously shown that *E. coli* RNA polymerase recognizes specific initiation sites on SV40 DNA. Except for one of them, determination of the location of these sites on the SV40 DNA map is only approximate. No specific termination site for transcripts has been identified, and consequently RNA polymerase generates a heterogeneous population of molecules. The size of some of these RNA molecules is several times the length of the viral genome.

We have shown that, after binding of the *E. coli* RNA polymerase to SV40 DNA, it was possible to cleave such transcriptional complexes with "single-cut" restriction endonucleases (Bam H_I, Eco R_I, Hpa II). The addition of ribonucleotide triphosphates to these linearized complexes leads to the synthesis of defined species of RNA which can be analyzed by electrophoresis. The determination of the size of each of these RNAs together with the assignment of the DNA strand on which they are transcribed will allow the precise mapping of the various RNA promoters (Reuveni, Lavialle, Salzman).

Transcriptional Properties of SV40 Nucleoprotein Cores

Simian Virus 40 (SV40) provides an excellent model system for investigating the process of transcriptional regulation in eukaryotic cells. Many structural aspects of viral transcriptional complexes and mRNA molecules have been determined. In addition, the entire nucleotide base sequence of SV40 DNA has been determined. Similar to most eukaryotic chromatin, during the SV40 infection cycle, cellular histones H_2A, H_2B_1, H_3 and H_4 are bound to the viral DNA. Late in the infection cycle, histone H_1 also becomes associated with the bulk of the viral chromatin in a stable nucleoprotein complex. Just prior to encapsidation, the SV40 nucleoprotein complex undergoes a redistribution of proteins and histone H_1 is replaced by viral proteins. Since binding of H_1 on chromatin may suppress transcription of DNA sequences, we were interested in determining if the viral proteins also acted to modify or regulate the process of RNA synthesis. Such regulatory properties could be important in a clearer understanding of the process of "early" mRNA synthesis during infection of the host cell. In view of the close similarity between this SV40 DNA-histone-viral protein nucleoprotein complex and cell chromatin structure, such studies would also be of interest in a more complete understanding of the general problem of transcriptional regulation in eukaryotic cells.

A nucleoprotein complex can be isolated from purified SV40 virions under very mild, physiological conditions. These nucleoprotein cores are potentially active transcriptional complexes. These core complexes do not contain endogenous RNA polymerase activity; however, 95-100% are able to form active transcriptional complexes. The transcriptional activity of those complexes is extremely high and is similar to the activity obtained with purified SV40 Form 1 DNA. This observation contrasts with published data, in which the level of transcription of nucleoprotein complexes (SV40 DNA-Histone) is generally less than 20% of the activity of deproteinized SV40 DNA. The results suggest that the viral proteins may play a role in the "activation" of the nucleohistone complex. Analysis of the composition and structure of the SV40 nucleoprotein core are being carried out and should allow an understanding of the unexpectedly high efficiency of the SV40 core as a template for RNA synthesis. (Brady, Lavialle, Salzman)

Parvoviruses

Kilham Rat Virus

The specific biochemical mechanisms involved in the replication of the autonomous parvovirus, KRV, has been examined in a rat nephroma cell line. The virus, isolated originally from a rat sarcoma, contains one molecule of linear, single-stranded DNA and three capsid proteins. Little information is available about replication of this single-stranded DNA virus in a eukaryotic cell or on the transcription of this DNA to make viral proteins. It was found that the virion KRV-DNA can self prime in vitro to synthesize the double-stranded replicative intermediate.

To define the structure of this self priming terminus, which is important in DNA replication, the nucleotides in the 3' DNA terminus have been sequenced. The DNA structure which can be deduced from the sequence contains a terminal hairpin. This is consistent with the finding that DNA replication is a self priming reaction.

It had previously been found that in an infected cell, two KRV specific mRNAs are synthesized. The DNA which is homologous to the major 21S viral mRNA has been mapped using both the "Southern" blotting technique and measurement of the "R loops" seen in the electron microscope. Inhibition studies suggest that these KRV mRNAs are synthesized by cellular RNA polymerase II. Preliminary studies also show that there are at least three virus induced proteins synthesized during infection.

Adeno Associated Viruses

The main objectives in studying defective human parvoviruses (AAV) are to define specific biochemical mechanisms used in synthesizing their DNA, RNA and proteins, to identify and characterize the helper virus-mediated step(s) required for their replication, to relate biochemical findings to normal cellular processes, and to determine conditions for selective interference of both AAV and Helper virus (adenoviruses, herpesviruses) infection.

Factors Which Confer Permissivity on Defective AAV

Human adenovirus (Ad) serotypes provide an early factor(s) that is necessary for adenovirus-associated virus (AAV) multiplication in human cell lines. However, little if any AAV production occurs in primary African green monkey kidney (AGMK) cells co-infected with AAV and a helper human Ad (non-permissive infection) unless cells are additionally infected with SV_{40} (permissive infection). To determine the basis of the host restriction of AAV replication in AGMK cells, AAV DNA, RNA and protein synthesis were analyzed under various conditions of infection. Hybridization reactions revealed no detectable AAV-specific DNA or RNA in infections with AAV alone or in combination with SV_{40}. In co-infections with AAV and Ad_5 or Ad_7, the synthesis of both AAV- and Ad-specific DNA and RNA occurred without a significant rise in titre of either virus. During non-permissive infection, however, AAV DNA synthesis was abnormal in that an expected accumulation of single-stranded progeny molecules was not observed. Finally, although intact 20S AAV transcripts were present in the cytoplasm of AGMK cells during non-permissive infection (in amounts ranging from 50 to 80% of that found during permissive infection), AAV-specific polypeptides were not demonstrable by polyacrylamide gel electrophoresis. Taken together, these experiments indicate that the host restriction of AAV replication in AGMK cells is exerted at the level of translation of the single AAV messenger RNA. In addition, it appears that one or more of the AAV polypeptides specified by this message is required for the production of single-stranded AAV progeny DNA. The fact that coinfection with a simian adenovirus, SV15, permits complete replication of both Ad and AAV (analogous to coinfection with

SV_{40}) strongly suggests that an Ad(SV15) gene product also exerts a regulatory effect on expression of the AAV mRNA (as well as several late human Ad mRNAs) in AGMK cells. Isolation and characterization of this gene product should be relevant to possible approaches for selectively interfering with virus infection. (Buller, Sebring, Rose)

AAV DNA Replication

Further understanding of mechanisms involved in DNA replication was obtained by studying AAV DNA synthesis in cell-free extracts. Extracts of nuclei from KB cells doubly infected with adenovirus-associated virus type 2 (AAV) and adenovirus type 2 (Ad) were examined for their ability to synthesize various molecular forms of AAV DNA. It was found that replicating AAV DNA molecules elongate to yield genome length hairpins or linear, open-ended duplexes. Both plus and minus DNA strands serve as templates for this synthesis which apparently does not reinitiate new rounds in vitro. Replicating Ad DNA molecules also could be identified in these extracts, but incorporation of [^{3}H]TTP into Ad DNA was diminished 80-90% in coinfections with AAV as compared to nuclei extracts prepared from cells infected with Ad alone. These results confirm our previous in vivo studies which indicated that a self-priming mechanism is involved in the replication of AAV DNA, studies that represented the first reported evidence for this mode of initiating DNA synthesis. Investigations with extracts will continue with the objective of identifying and characterizing enzymatic and regulatory components that participate in viral and cellular DNA replication. (Sebring, Buller, Rose)

Adenoviruses

The major objective of these studies has been the application of physical and biochemical techniques to map and to define the structure of specific genetic regions in the genomes of both oncogenic and non-oncogenic human adenoviruses, with the ultimate goals of (1) relating these sequences to those biochemical activities which permit these viruses to influence or gain control of cellular functions (e.g., cell lysis and transformation) and (2) of defining basic mechanisms for regulation of genetic expression (e.g., initiation of DNA synthesis). A number of unique features have been described which may have implications in viral replication or carcinogenesis or both.

DNA Terminal Proteins

The DNA of adenoviruses is normally isolated as a linear duplex molecule of discrete size using standard procedures of extraction with protealytic enzymes, phenol and SDS. Recently, it has been possible to demonstrate forms of DNA that are either circular or oligomeric by using procedures which do not involve proteolytic enzymes. In these latter cases, the DNA molecules are always joined at their ends, and it appears that either end of a molecule may interact with the other end of the same molecule or with either end of another molecule. The circles and oligomers are resistant to treatment with 4M guanidinium chloride, 4M urea, formimide, sarkosyl or mercaptoethanol. Treatment with proteases,

however, rapidly converts the circles and oligomers to linear double-stranded monomers, as does treatment with SDS. It had been previously proposed that there is a protein attached to each end of the DNA molecule and that this protein is responsible for the formation of the observed complexes. Subsequent studies have clearly demonstrated the presence of a 55,000 dalton protein very tightly linked to the DNA molecule. Although the function of the bound protein is unknown, it has been proposed that it may be involved in DNA replication by allowing initiation or completion of the 5' ends of the progeny strands. It has also been suggested that the protein may have a structural role by circularizing the DNA within the virus particle, may protect the DNA from exonuclease digestion or may act as an endonuclease in a hairpin model of DNA replication. Furthermore, it has been recently demonstrated that the efficiency of transfection with the Ad 5 DNA protein complex is about 100-fold higher than that of pronase-treated Ad 5 DNA.

Brown et al. (Journal of Virology 16, 366) first demonstrated that these DNA-protein complexes did not migrate into agarose gels during electrophoresis. This observation provided the basis for a useful means for detecting not only the presence or absence of terminal protein components but the identification and purification of terminal DNA sequences as well. We have fractionated the Ad 2 DNA-protein complex on hydroxylapatite columns and analyzed the DNA eluate by agarose gel electrophoresis. Although the initial DNA-protein complexed did not migrate into agarose gels, >75% of the initial DNA molecules in the DNA eluate were now able to enter the gels in spite of the absence of protease or SDS treatment. When the protein in the DNA eluate was labeled in vitro with ^{125}I, protein was not seen associated with the DNA that migrated into the gels. When the DNA eluate was examined in the electron microscope, DNA was found to be essentially linear. The re-addition of a DNA-free protein fraction to the DNA eluate produced circularization of about 20% of these linear molecules. We conclude that circularization of the adenovirus DNA-protein complex as well as its inability to migrate into agarose gels is likely due to an aggregate of a virion component(s) in addition to the covalently-attached 55K protein previously described.

Significant quantitative differences were noted when Ad 18, a highly oncogenic serotype, was extracted with 4M guanidinium chloride and the DNA-protein complexes assayed in similar fashion. Purified virions of adenovirus serotype 2 and 18 were applied to gradients containing guanidinium, peak fractions were pooled and the DNA-protein complexes dialyzed and compared. 35-55% of the molecules from each serotype appeared circular in the electron microscope. While the DNA-protein complexes from serotype 2 were effectively bound to glass fiber filters (99%) under the conditions employed, over 45% of the guanidinium purified material isolated from serotype 18 passed unimpeded through the filter under identical conditions. Of some interest was the fact that nearly all molecules in this fraction were circular. Bound material could be effectively released from filters with 1% SDS or pronase digestion. DNA molecules from either serotype when extracted with proteolytic enzymes did not bind to filters. Approximately half of the guanidinium purified material from serotype 18 was shown to enter

agarose gels upon electrophoresis. The structural implications of this population of molecules which apparently does not bind to glass fiber filters and which easily penetrates agarose gels, yet retains the ability to circularize, is of considerable biological interest. (Garon, Rose)

Pox Viruses

Vaccinia

Our primary objective is to determine the molecular events that lead to the selective transcription of DNA and subsequent processing and translation of messenger RNA (mRNA). Poxviruses provide a unique and important system for such studies since the enzymes needed for transcription and mRNA modification are packaged within the core of infectious virus particles. A single enzyme complex containing three activities - RNA triphosphatase, RNA guanylyltransferase, and RNA (guanine-7-)methyltransferase - has been purified from vaccinia virus. Acting consecutively, the individual activities in the complex are capable of modifying the 5'-end of nascent RNA molecules to form a cap structure in vitro. Extracts from uninfected human cells were shown to carry out a similar set of reactions, however, three separate enzymes were found instead of a single complex. The donor and acceptor substrate specificities of the different viral and cellular enzymes suggested mechanisms used for processing mRNA in vivo. Evidence for temporal control of vaccinia virus gene expression at the transcriptional level has been obtained by DNA:RNA hybridization studies and by translation of viral mRNA in a cell-free protein synthesizing system. Transcriptional and translational maps of the vaccinia virus genome are being prepared using DNA fragments, produced by restriction endonuclease digestion, that have been cloned in phage lambda using approved DNA recombinant technology.

Major Findings

1. Post-transcriptional modification of mRNA

A. Isolation and characterization of viral capping and methylating enzymes.

Members of this research group previously identified the 5'-terminal cap structure $m^7G(5')pppN$ consisting of a 7-methylguanosine (m^7G) linked by a 5' to 5' triphosphate bridge to one or two consecutive 2'-0-methylated ribonucleosides (N^m) present in viral and cellular mRNAs. The mechanism of cap formation was determined by purifying the appropriate enzymes from vaccinia virus. The latter, included (i) an enzyme complex containing both mRNA guanylyltransferase, which is the capping enzyme, and mRNA(guanine-7-) methyltransferase and (ii) a separate mRNA (nucleoside-2'-)-methyltransferase. Further studies of the purified enzyme complex during the past year revealed a third activity, RNA triphosphatase. On a molar basis, the RNa triphosphatase is nearly 100-fold more active than the associated mRNA guanylyltransferase. Consequently, both triphosphate and diphosphate-ended RNAs are capped at similar rates and modification of the 5'-end of incomplete or nascent transcripts occurs in the following order:

$$pppN_1\text{-}N_2\ldots \rightarrow ppN_1\text{-}N_2\ldots + Pi \quad \text{(i)}$$

$$GTP + ppN_1\text{-}N_2\ldots \rightleftarrows G(5')pppN_1\text{-}N_2\ldots + PPi \quad \text{(ii)}$$

$$AdoMet + G(5')pppN_1\text{-}N_2\ldots \rightarrow m^7G(5')pppN_1\text{-}N_2\ldots + AdoHcy \quad \text{(iii)}$$

$$AdoMet + m^7G(5')pppN_1\text{-}N_2\ldots \rightarrow m^7G(5')pppN_1^m\text{-}N_2\ldots + AdoHcy \quad \text{(iv)}$$

(Venkatesan, Moss)

B. Isolation and characterization of cellular capping and methylating enzymes.

Previously, we detected capping activity in crude extracts prepared from HeLa cell nuclei. During the past year, this enzyme has been purified approximately 1,000-fold and extensively characterized particularly with respect to its donor and acceptor substrate specificities. This analysis has provided important information regarding the mechanism of capping cellular RNA. Of a variety of potential donor molecules, only GTP and ITP were utilized by the purified enzyme. These results indicated that the mRNA guanylyltransferase recognizes the oxygen on the purine ring of GTP, discriminates between ribose and deoxyribose sugars, and requires a triphosphate. The inability to use m^7GTP as a donor indicates that methylation occurs after capping. With regard to acceptor molecules, the enzyme is specific for a diphosphate-ended oligo- or polyribonucleotide. The inability to efficiently cap triphosphate-ended acceptors is explained by the absence of an associated RNA triphosphatase. In that respect, and also by the absence of an associated mRNA(guanine-7-)methyltransferase, the HeLa cell capping enzyme differs from the one isolated from vaccinia virus. This result is consistent with our previous purification of a specific HeLa cell mRNA (guanine-7-)methyltransferase. Although the possibility of a pre-transcriptional capping mechanism has been considered by others, our finding that the minimum length acceptor is a dinucleotide such as ppGpC makes this mechanism unlikely. In fact, the Km for a dinucleotide is higher than for a polymer indicating that a longer oligonucleotide or short polyribonucleotide is a better acceptor. We suggest that in vivo, an RNA triphosphatase removes the third phosphate from the 5'-end of short unfinished primary transcripts and that they are then capped by the enzyme that we have described. If this enzyme also caps RNA at sites of cleavage, then additional kinases capable of forming diphosphate-ends must exist. (Venkatesan, Moss)

c. Coupled transcription and modification

One of the major advantages of working with vaccinia virus is that all of the enzymes necessary for synthesis and modification are contained within the purified virus particle. After irradiating purified vaccinia virus particles with ultraviolet light to form pyrimidine dimers in the DNA, only short abortive transcription products were made. Analysis of these products indicated, however, that they were capped, methylated and polyadenylylated. These results suggested to us that neither completion of an RNA chain nor processing from a polycistronic precursor was required for modification of either end of the RNA. The presence of the poly(A)

tract at the 3'-end of the short transcript further suggested that a slow-down or cessation of transcription, rather than a specific 3'-terminal sequence, serves as a signal for polyadenylylation. (Gershowitz, Moss)

2. Organization and regulation of the vaccinia virus genome

A. In vitro translation of immediate early, early and late classes of RNA from vaccinia virus infected cells

Cytoplasmic RNA, isolated at various times after vaccinia virus infection, was translated in a message-dependent cell-free system prepared from rabbit reticulocytes. When programmed with RNA extracted at 2 hr after infection, early viral proteins were made and formation of cellular proteins was diminished. Primarily late proteins were synthesized using RNA extracted at 4 or more hours after infection, suggesting that the switch in protein synthesis is regulated principally by changes in RNA concentration rather than by modification of the translation apparatus of the cell. Immediate early RNA was obtained by infecting cells in the presence of inhibitors of protein synthesis. Comparisons between immediate early and early gene products did not reveal a class of early genes that require protein synthesis for expression. On the contrary, seven polypeptides, of which a 28,000 dalton species was most prominent, were synthesized in relatively greater amounts with immediate early RNA than with early RNA. This result suggests the possibility that expression of certain immediate early genes is regulated by a feed-back mechanism. (Cooper, Moss)

B. Analysis of the terminal repetition within the vaccinia virus genome

Previously we reported that a 3.54 μm duplex was formed by annealing the two ends of the vaccinia virus genome. This inverted terminal repetition was estimated to be about 10,000 nucleotide base pairs long. To further characterize this structural feature, restriction maps were made of the ends of the genome using the following enzymes: Eco RI, Hpa II, and Hind II. To accomplish this, we developed a novel mapping method, involving use of "nick translated" probes prepared from the terminal fragment, to order partial digestion products. From this study, we concluded that identical restriction sites are present at the 2 ends of the genome for approximately 10,000 nucleotides confirming our previous electron microscopic analysis and work of other investigators.

To determine whether the inverted terminal repetition is transcribed in vivo, ^{32}P-labeled cytoplasmic RNA was obtained from infected cells. This RNA was then annealed to restriction fragments, prepared from the terminal repetition and adjacent regions of the DNA that were immobilized to a nitrocellulose sheet. We found that early RNA hybridized to the repetition except for the terminal 3,000 nucleotide base pairs indicating that it is indeed transcribed. (Barbosa, Moss)

C. Formation of recombinant DNA molecules containing portions of the vaccinia virus genome.

DNA recombinant technology developed within the past few years followed by a relaxation of the guidelines for carrying out such research

has allowed us to clone portions of the vaccinia virus genome in phage lambda. Our initial approach has been to clone successive Eco RI fragments of DNA starting from one end of the genome. Thus far, this has included a 9,500 nucleotide base-pair segment containing most of the terminal repetition, a 6,500 nucleotide base-pair segment containing unique sequences adjacent to this, and the next 4,000 nucleotides base-pair segment. In order to clone the end piece of DNA, it was first necessary to remove the terminal cross-link with a single-strand specific nuclease and then add synthetic Eco RI linkers. Each of the recombinant DNA clones were identified by restriction endonuclease digestion and gel electrophoresis. Studies are now in progress using the cloned genome fragments to obtain a transcription and translation map. (Wittek, Chan, Moss)

3. Action of specific antiviral agents

Isatin-β-thiosemicarbazone is known to specifically inhibit vaccinia virus replication at a late stage. At 6 hr after infection, viral protein synthesis was inhibited by about 95%. We confirmed that a portion of the virus-specific RNA appears to be degraded (B. Woodson and W. K. Joklik, 1965, Proc. Natl. Acad. Sci., USA 54: 946-953). Nevertheless, the amount of viral RNA that was capped, properly methylated, and polyadenylylated, was reduced by only about 50%. Moreover, RNA from IBT-treated cells stimulated cell-free protein synthesis to one-half the level obtained with RNA from control cells. Polyacrylamide gel electrophoretic analysis further demonstrated that RNA from IBT-treated cells was translated into late viral proteins in vitro. Thus, it seems possible that the inhibition of protein synthesis in IBT-treated cells does not result entirely or directly from either an inhibition of mRNA synthesis or from a depletion of mRNA caused by accelerated degradation. An alternative possibility, that accelerated degradation is secondary to a more immediate effect of the drug on protein synthesis, was considered. (Cooper, Moss)

Structural Studies of the Viral Genome

Vaccinia DNA has covalently-linked-complementary (CLC) ends which define each end of the physical map of the genome. These CLC end segments can be identified in each set of segments that are produced by a particular restriction enzyme. The set of segments is alkali denatured, neutralized and passed through a BND-cellulose column from which only the CLC segments elute as duplex chains. The end segments have been identified for several restriction enzymes such as Sal I, Hind III, Kpn I and Xho I. For Sal I, the end segments are identical and small with a mass of 2.1×10^6 daltons. For Xho they are larger (both 3.9×10^6) and for Hind III they are larger still (17.6 and 13.5×10^6).

The restriction enzymes listed above as well as several others are used to digest vaccinia DNA to obtain unique DNA segments which are separated by agarose electrophoresis. To obtain a physical map, segments produced by one enzyme are isolated from agarose and digested by a second enzyme to find overlapping regions. The end segments described here serve as reference points from which the maps are continued. The Sal I and Hind III map of vaccinia strain WR are now almost completely known. Kpn and Xho maps are also near completion.

A particular method which has helped to generate reproducible partial DNA segments involves restriction enzyme digestion in the presence of actinomycin D. The original observation that the drug blocked complete digestion with Hind III has been extended to 3 other restriction enzymes, Sal I, Xho and Kpn. None of these digests produce as long a partial as the one half length vaccinia DNA molecule generated with Hind but they do contain several smaller linked segments which help to establish the map.

Initial experiments designed to locate the origin of replication involve pulse labeling HeLa cells with ^{3}H-thymidine after infection with high multiplicities of vaccinia virus. A peak of synthetic activity occurs 2.5 hours after infection. From 2.5 to 3.5 hours after infection alkaline and neutral sucrose gradients of cytoplasmic (viral) DNA reveal a spectrum of molecular sizes with about 10 - 20% of the DNA in full length molecules. These DNA species will be examined in a Dintzis (Proc. Natl. Acad. Sci. 47: 247, 1961) type experiment.

Several experimental techniques have evolved during the year. The method of Vogelstein and Gillespie (Proc. Natl. Acad. Sci. 76: 615, 1979) for extracting DNA segments from agarose with glass powder has been modified so that it is now possible to routinely recover intact, 70% or more of µg amounts of large vaccinia DNA segments. Also, poly rC columns have been used to hybridize segments which contain short stretches of GC rich regions in duplex DNA. Using this column technique, we have found that the largest Sal I segment binds to the column. Since GC rich regions may provide a structural basis for a signal element, the Sal I segment will be digested with other enzymes to localize the GC region. Finally, we have started to use malachite green columns to fractionate sheared vaccinia DNA (6 X 10^{6} daltons) according to base composition. Those fragments with the least affinity for the column have the highest average GC content. They will be radiolabeled and hybridized to Sal I digests to identify those segments from which they originated. (De Filippes)

Molluscum contagiosum

Molluscum contagiosum virions were isolated from clinically typical skin lesions and the DNA extracted and characterized using techniques of electron microscopy and agarose gel electrophoresis. The structural characteristics determined in these studies tend to place the MCV genome among other members of the poxvirus group in terms of genome molecular weight and organization. The apparent denaturability of the MCV genome into a continuous, single-stranded circle would point to the presence of terminal cross-links in the DNA molecule. So far, this structural arrangement appears to be unique to members of the poxvirus group. The objective of these studies has been to define structural features of these molecules and to relate them to the biochemical events involved in the replication and growth of this virus in the cells they infect.

Extracts from molluscum contagiosum lesions when stained with sodium phosphatungstate and examined in the electron microscope appears to

contain many structurally mature virus particles of both C and M forms. However, the virus has not been successfully propagated in the laboratory. Molecular information about this virus has been limited to description of size, shape and composition and has been difficult to obtain. The structural characteristics determined in our initial studies (Virology 81: 247) tend to place the MCV genome among other members of the poxvirus group in terms of molecular weight and organization. The average molecular weight of MCV-DNA was calculated to be 118 X 10^6 and appeared to be extremely sensitive to both mechanical shear and nuclease damage. Single-stranded circles measuring twice the length of linear molecules were observed following high levels of denaturation. This now appears to be a generalized feature of all poxvirus DNAs so far examined. The biological function, if any, of the unique terminal-cross-links is unknown. Also of special interest was the fact that the most easily denaturable regions of the MCV genome (and presumably that of the highest AT content) appeared to be the end 19% of the DNA molecule. That the MCV genome differs markedly from that of vaccinia is clear from the visual denaturation profiles and from the restriction endonuclease cleavage patterns obtained in our initial studies. Further differences were noted in the extractability of the various poxvirus genomes. Procedures used for successfully extracting and purifying the DNA molecules from one viral genome proved completely unsuitable for the other.

Previously, little was known about whether most isolates belonged to the same or different MCV strains or whether a given strain was always isolated from similar clinical sources. A survey of the restriction endonuclease fragment patterns of several independent MCV isolates was initiated. Lesions obtained from individual patients were never pooled but rather purified, extracted, and assayed as a single isolate. Initially, eleven such isolates were processed. In these studies, lesions isolated from various sites on an individual patient appeared to contain virus whose DNA showed identical gel patterns. Furthermore, virus was isolated from the lesions of patients' relatives which, when assayed by the above restriction endonuclease procedure, showed gel patterns identical to those of the patients themselves. Interestingly, only 3 characteristically frequent patterns were observed among eleven independent isolates. Restriction endonuclease digestion produced 11 to 20 fragments ranging in size from 4 X 10^5 to 31 X 10^6 daltons depending on the virus isolate. Mixing experiments showed comigration of some fragments in all patterns. Mixing experiments showed comigration of some fragments in all patterns. Although comigration of DNA fragments does not necessarily mean base sequence identity, extensive comigration would imply significant genetic or organization similarity among the viral genomes. Direct comparisons of these gel purified fragments by hybridization or heteroduplex analysis have not been attempted due to limits in the quantity of viral DNA obtainable from each MCU isolate.

Although we have not collected a large enough body of data to make any reasonable assessment of the relationship between the clinical characteristics of the disease and the restriction endonuclease cleavage patterns, it may be possible once the data are accumulated, to relate tehse observations and to develop a useful molecular epidemiological

scheme. Such a scheme would make possible rapid identification and classification of MCV isolates without the necessity of propagating the virus in the laboratory. Furthermore, since the degree of sensitivity of such a system is limited to only the number of cleavage sites recognized by a given restriction enzyme, other available enzymes may easily be substituted.

We propose to continue to define structural features of these poxvirus DNA molecules with the aim of (1) comparing genetic variation among members of this group, and of (2) relating these features to those biochemical events which are involved in the replication of these agents.

Honors and Awards

Dr. Norman P. Salzman continued to serve on the Editorial Board of the Journal of Virology and on the Editorial Advisory Board, Biochemistry, and Scientific Board of the Coordinating Council for Career Research. He serves as Professorial Lecturer, Georgetown University School of Medicine, and was an invited participant and Session Chairman at the EMBO/FEBS Workshop on Gene Structure and Formation of RNA of Viruses and Cells.

Dr. Claude Garon received the N.I.H. Merit Award.

Dr. James Rose was an invited participant at the EMBO/FEBS workshop.

Dr. Bernard Moss continued to serve as associate editor of Virology and on the editorial boards of the Journal of Virology, of Antibiotics and Chemotherapy, and of Intervirology. In addition, he received the PHS commendation Medal, was an invited principal speaker at the Society for General Microbiology (U.K.) Meeting, was a Session Chairman at an International Conference on Transmethylation, was invited speaker at the EMBO/FEBS workshop on Gene Structure and Formation of RNA of Viruses and Animal Cells, and served on a Poxvirus Study Group for the World Health Organization.

Dr. Riccardo Wittek, a guest investigator in the LBV, received the Forderungspreis which is awarded annually to the outstanding young microbiologist in Switzerland, and also served on a Poxvirus Study Group for the World Health Organization.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00123-13 LBV

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Messenger RNA: Regulation of Synthesis, Modification and Translation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: Bernard Moss, Medical Director, LBV, NIAID

Other:	Sundararajan Venkatesan	Senior Staff Fellow, LBV, NIAID
	Ernest Barbosa	Research Associate, LBV, NIAID
	Steven Langberg	Staff Fellow, LBV, NIAID
	Jonathan Cooper	Visiting Fellow, LBV, NIAID
	Bahige Baroudy	Visiting Fellow, LBV, NIAID
	Riccardo Wittek	Guest Investigator, LBV, NIAID

COOPERATING UNITS (if any)

Ehud Katz, Dept. of Virology, Hebrew University Medical Center, Jerusalem, Israel

LAB/BRANCH
Laboratory of Biology of Viruses

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Our primary objective is to determine the molecular events that lead to the selective transcription of DNA and subsequent processing and translation of messenger RNA (mRNA). Poxviruses provide a unique and important system for such studies since the enzymes needed for transcription and mRNA modification are packaged within the core of infectious virus particles. A single enzyme complex containing three activities - RNA triphosphatase, RNA guanylytransferase, and RNA (guanine-7-)methyltransferase - has been purified from vaccinia virus. Acting consecutively, the individual activities in the complex are capable of modifying the 5'-end of nascent RNA molecules to form a cap structure in vitro. Extracts from uninfected human cells were shown to carry out a similar set of reactions, however, three separate enzymes were found instead of a single complex. The donor and acceptor substrate specificities of the different

viral and cellular enzymes suggested mechanisms used for processing mRNA in vivo. Evidence for temporal control of vaccinia virus gene expression at the transcriptional level has been obtained by DNA:RNA hybridization studies and by translation of viral mRNA in a cell-free protein synthesizing system. Transcriptional and translational maps of the vaccinia virus genome are being prepared using DNA fragments, produced by restriction endonuclease digestion, that have been cloned in phage lambda using approved DNA recombinant technology.

Major Findings

1. Post-transcriptional modification of mRNA

A. Isolation and characterization of viral capping and methylating enzymes - Members of this research group previously identified the 5'-terminal cap structure $m^7G(5')pppN$ consisting of a 7-methylguanosine (m^7G) linked by a 5' to 5'triphosphate bridge to one or two consecutive 2'-O-methylated ribonucleosides (N^m) present in viral and cellular mRNAs. The mechanism of cap formation was determined by purifying the appropriate enzymes from vaccinia virus. The latter, included (i) an enzyme complex containing both mRNA guanylyltransferase, which is the capping enzyme, and mRNA(guanine-7-)methyltransferase and (ii) a separate mRNA (nucleoside-2'-) methyltransferase. Further studies of the purified enzyme complex during the past year revealed a third activity, RNA triphosphase. On a molar basis, the RNA triphosphatase is nearly 100-fold more active than the associated mRNA guanylytransferase. Consequently, both triphosphate and diphosphate-ended RNAs are capped at similar rates and modification of the 5'-end of incomplete or nascent transcripts occurs in the following order:

$$pppN_1\text{-}N_2\ldots \rightarrow ppN_1\text{-}N_2\ldots\ldots\ldots +Pi \qquad (i)$$

$$GTP+ppN_1\text{-}N_2\ldots \rightleftarrows G(5')pppN_1\text{-}N_2\ldots\ldots +PPi \qquad (ii)$$

$$AdoMet+G(5')pppN_1\text{-}N_2\ldots\ldots \rightarrow m^7G(5')pppN_1\text{-}N_2\ldots +AdoHcy \qquad (iii)$$

$$AdoMet+m^7G(5')pppN_1\text{-}N_2\ldots\ldots \rightarrow m^7G(5')pppN_1^mN_2\ldots +AdoHcy \qquad (iv)$$

B. Isolation and characterization of cellular capping and methylating enzymes - Previously, we detected capping activity in crude extracts prepared from Hela cell nuclei. During the past year, this enzyme has been purified approximately 1,000-fold and extensively characterized particularly with respect to its donor and acceptor substrate specificities. This analysis has provided important information regarding the mechanism of capping cellular RNA. Of a variety of potential donor molecules, only GTP and ITP were utilized by the purified enzyme. These results indicated that the mRNA guanylytransferase recognizes the oxygen on the purine ring of GTP, discriminates between ribose and deoxyribose sugars, and requires a triphosphate. The inability to use m^7GTP as a donor indicates that methylation occurs after capping. With regard to acceptor molecules, the enzyme is specific for a diphosphate-ended oligo-or polyribonucleotide. The inability to efficiently cap triphosphate-ended acceptors is explained by the absence of an associated RNA triphosphatase. In that respect and also by the absence

of an associated mRNA(guanine-7-)methyltransferase, the Hela cell capping enzyme differs from the one isolated from vaccinia virus. This result is consistent with our previous purification of a specific Hela cell mRNA (guanine-7-)methyltransferase. Although the possibility of a pre-transcriptional capping mechanism has been considered by others, our finding that the minimum length acceptor is a dinucleotide such as ppGpC makes this mechanism unlikely. In fact, the Km for a dinucleotide is higher than for a polymer indicating that a longer oligonucleotide or short polyribonucleotide is a better acceptor. We suggest that in vivo, an RNA triphosphate removes the third phosphate from the 5'-end of short unfinished primary transcripts and that they are then capped by the enzyme that we have described. If this enzyme also caps RNA at sites of cleavage, then additional kinases capable of forming diphosphate-ends must exist.

C. Coupled transcription and modification - One of the major advantages of working with vaccinia virus is that all of the enzymes necessary for synthesis and modification are contained within the purified virus particle. After irradiating purified vaccinia virus particles with ultraviolet light to form pyrimidine dimers in the DNA, only short aborative transcription products were made. Analysis of these products indicated, however, that they were capped, methylated and polyadenylylated. These results suggested to us that neither completion of an RNA chain nor processing from a polycistronic precursor was required for modification of either end of the RNA. The presence of the poly(A) tract at the 3'-end of the short transcript further suggested that a slow-down or cessation of transcription, rather than a specific 3'-end terminal sequence, serves as a signal for polyadenylylation.

2. Organization and regulation of the vaccinia virus genome.

A. In vitro translation of immediate early, early and late classes of RNA from vaccinia virus infected cells - Cytoplasmic RNA, isolated at various times after vaccinia virus infection, was translated in a message-dependent cell-free system prepared from rabbit reticulocytes. When programmed with RNA extracted at 2 hr after infection, early viral proteins were made and formation of cellular proteins was diminished. Primarily late proteins were synthesized using RNA extracted at 4 or more hours after infection, suggesting that the switch in protein synthesis is regulated principally by changes in RNA concentration rather than by modification of the translation apparatus of the cell. Immediate early RNA was obtained by infecting cells in the presence of inhibitors of protein synthesis. Comparison between immediate early and early gene products did not reveal a class of early genes that require protein synthesis for expression. On the contrary, seven polypeptides, of which a 28,000 dalton species was most prominent, were synthesized in relatively greater amounts with immediate early RNA than with early RNA. This results suggests the possibility that expression of certain immediate early genes is regulated by a feed-back mechanism.

B. Analysis of the terminal repetition within the vaccinia virus genome - Previously we reported that a 3.54μm duplex was formed by annealing the two ends of the vaccinia virus genome. This inverted terminal repetition

was estimated to be about 10,000 nucleotide base pairs long. To further characterize this structural feature, restriction maps were made of the ends of the genome using the following enzymes: EcoRI, HpaII, and HindIII. To accomplish this we developed a novel mapping method, involving use of "nick translated" probes prepared from the terminal fragment, to order partial digestion products. From this study, we concluded that identical restriction sites are present at the 2 ends of the genome for approximately 10,000 nucleotides confirming our previous electron microscopic analysis and work of other investigators.

To determine whether the inverted terminal repetition is transcribed in vivo, ^{32}P-labeled cytoplasmic RNA was obtained from infected cells. This RNA was then annealed to restriction fragments, prepared from the terminal repetition and adjacent regions of the DNA that were immobilized to a nitrocellulose sheet. We found that early RNA hybridized to the repetition except for the terminal 3,000 nucleotide base pairs indicating that it is indeed transcribed.

C. Formation of recombinant DNA molecules containing portions of the vaccinia virus genome - DNA recombinant technology developed within the past few years followed by a relaxation of the guidelines for carrying out such research has allowed us to clone portions of the vaccinia virus genome in phage lambda. Our initial approach has been to clone successive EcoRI fragments of DNA starting from one end of the genome. Thus far, this has included a 9,500 nucleotide base-pair segment containing most of the terminal repetition, a 6,500 nucleotide base-pair segment containing unique sequences adjacent to this, and the next 4,000 nucleotides base-pair segment. In order to clone the end piece of DNA, it was first necessary to remove the terminal cross-link with a single-strand specific nuclease and then add synthetic EcoRI linkers. Each of the recombinant DNA clones were identified by restriction endonuclease digestion and gel electrophoresis. Studies are now in progress using the cloned genome fragments to obtain a transcription and translation map.

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Isatin-β-thiosemicarbazone is known to specifically inhibit vaccinia virus replication at a late stage. At 6 hr after infection, viral protein synthesis was inhibited by about 95%. We confirmed that a portion of the virus-specific RNA appears to be degraded (B. Woodson and W.K. Joklik, 1965, Proc. Nat. Acad. Sci., USA 54, 946-953). Nevertheless, the amount of viral RNA that was capped, properly nethylated, and polyadenylylated, was reduced by only 50%. Moreover, RNA from IBT-treated cells stimulated cell-free protein synthesis to one-half the level obtained with RNA from control cells. Polyacrylamide gel electrophoretic analysis further demonstrated that RNA from IBT-treated cells was translated into late viral proteins in vitro. Thus, it seems possible that the inhibition of protein synthesis in IBT-treated cells does not result entirely or directly from either an inhibition of mRNA synthesis or from a depletion of mRNA caused by accelerated degradation. An alternative possibility, that accelerated degradation is secondary to a more immediate effect of the drug on protein synthesis, was considered.

Publications

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00124-10 LBV

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Replication of the Parvovirus, KRV

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: Dr. Lois A. Salzman Research Chemist LBV NIAID

Other: Dr. Tonu Wali, Visiting Fellow LBV NIAID
Ms. Phyllis Fabisch, Microbiologist LBV NIAID

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have been studying the specific biochemical mechanisms involved in the replication of the autonomous parvovirus, KRV in a rat nephroma cell line. The virus, isolated originally from a rat sarcoma, contains one molecule of linear, single-stranded DNA and three capsid proteins. Little information is available about replication of a single-stranded DNA virus in a eukaryotic cell or on the transcription of this DNA to make viral proteins. We have demonstrated that the virion KRV-DNA can self prime in vitro to synthesize the double-stranded replicative intermediate. In an attempt to resolve this self priming terminus, so important in DNA replication, we have sequenced the nucleotides in the 3' DNA terminus which probably also contains the in vitro origin of DNA replication.

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We have previously found that in an infected cell two KRV specific mRNAs are synthesized. We have mapped the major 21S viral mRNA on the viral DNA molecule using both the "Southern" blotting technique and measurement of the "R loops" seen in the electron microscope. Inhibition studies have shown that these KRV mRNAs are probably synthesized by cellular RNA polymerase II. Preliminary studies also show that there are at least three virus induced proteins synthesized during infection.

Project Description

Parvoviruses are the smallest DNA containing viruses found in vertebrate tissues. They infect a wide variety of species including man. The parvoviruses are classified as autonomous or defective depending on whether or not they require a helper virus for replication. The virions replicate best in rapidly dividing cells and produce a wide variety of effects on insects, animals and cells in culture. The feline parvovirus has been linked to the naturally occuring disease feline panleukopenia. Rodent parvoviruses can produce fatal infections and teratogenic effects in fetal and newborn hamsters. Parvoviruses are able to persist in a latent state and to inhibit homologous and heterlogous virus infection. The viruses can also exhibit antimitotic activity and interfere with natural or virus-induced tumorigenesis.

We have continued our studies of the parvovirus, KRV, because of its genetic simplicity and the small size of the virus gene. KRV like the other parvoviruses possesses a single-stranded DNA genome (1.6 x 106 daltons or 4500 to 5000 bases) with sufficient information to code for only a few proteins equvalent to about 150,000 daltons. The transcription process may be very simple and involve only one or two messenger RNAs. It is probable that the viral DNA replication and transcriptional components are carried out in large part by host functions. Thus, we potentially can gain insight into viral functions, host functions and the interaction between virus and host.

In the past year we have concentrated our efforts into studying further viral DNA replication, transcription of the viral genome and virus induced proteins. We have found that the single-stranded DNA from KRV can serve in vitro as a self-primer for the synthesis of a complementary linear viral DNA strand. A double stranded viral DNA molecule has been proposed as an intermediate in the replication of the viral genome. Little is known about the replication of single-stranded linear DNA in a eukaryotic cell. Because of the importance of the 3' terminus of the viral DNA in self priming viral DNA synthesis and possibly in transcription we sequenced the 3' terminal nucleotides. This analysis has lead us to propose a 3' terminal hairpin structure with secondary structures and the nucleotide sequence of the DNA which at least in vitro serves as the origin of replication of the complementary strand. We are currently sequencing the 5' terminus of the viral DNA which appears to have a unique structure and possibly an associated protein.

In our studies of the transcription of KRV, we have previously found

that two KRV-specific mRNAs are synthesized in infected RN cells. We have also found that only the viral DNA strand is transcribed. Two techniques have now been used to map these mRNAs to the viral genome. We have mapped the fragments of several restriction enzymes to the KRV viral genome. Using the mapped restriction fragments, the major cytoplasmic viral RNA (21 S) has been mapped to the viral genome using the "Southern" blotting technique. This has been complemented by Electron Microscopy of "R loops" or RNA-DNA duplexes. Both techniques indicate that the 21S viral mRNA maps from approximately 0.38 to 0.98 on the viral DNA strand.

Further studies are presently underway to determine if longer transcripts are present in the nucleus, and to try and determine if posttranscriptional processing occurs by simple cleavage or by "splicing". We are also trying to elucidate structural features of the KRV mRNA by assaying for RNA cap structures at the 5' end and to determine translatability of the KRV mRNAs in in vitro assays.

We have used nuclei isolated from infected rat nephroma cells to study the enzymes involved in viral specific transcription. Hybridization of RNA synthesized in isolated nuclei indicated that viral specific RNA synthesis started at 8 to 9 hours post infection. Viral specific RNA was inhibited by 0.1 g of amamitin per ml, suggesting that the viral genome is transcribed by cellular RNA polymerase II. The viral RNA synthesized in the isolated nuclei was also analyzed on sucrose gradinets. The KRV specific RNA varied in length from 26S (full DNA transcript) to 4S in length. We propose to further study the components involved in transcription of the viral genome after gentle disruption of the nuclei.

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4. Salzman, L.A., P. Fabisch. Nucleotide sequence of the self-priming 3'terminus of the single-stranded DNA extracted from the Parvovirus, Kilham Rat Virus. J. Virol. 30: 946-951 (1979).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00125-10 LBV

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Mechanisms of Viral DNA Replication, Transcription, and Integration

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Norman P. Salzman	Chief	LBV, NIAID
	Walter Bruszewski	Staff Fellow	LBV, NIAID
	Minou Bina-Stein	Senior Staff Fellow	LBV, NIAID
	Nancy Chiu	Staff Fellow	LBV, NIAID
	Yaffa Reuveni	Visiting Associate	LBV, NIAID
	John A. Thompson	Staff Fellow	LBV, NIAID
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	Christian Lavialle	Visiting Fellow	LBV, NIAID

COOPERATING UNITS (if any)

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Laboratory of Biology of Viruses

SECTION
Biochemical Virology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
9.0	1.0	4.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The structure of SV40 mRNA molecules has been determined by preparing cDNA copies of the viral mRNAs using reverse transcriptase. Characterization of transcripts formed when SV40 DNA is transcribed by purified RNA polymerases has provided the location of promoters of RNA transcription. The role of viral proteins as regulators of transcription has been studied by in vitro transcription of nucleoprotein complexes.

PHS-6040
(Rev. 10-76)

Papovaviruses

The current studies have tried to define some of the processes responsible for the formation of viral messenger RNA molecules. Thus far, these studies have provided the precise structures of the viral mRNA molecules, have defined the nature of the templates used for the synthesis of viral transcripts, have located promoters on the SV40 genome that are recognized by E. coli RNA polymerase and have compared SV40 DNA I and SV40 DNA I nucleoprotein complexes as templates for transcription.

The Structure of Early and Late Viral mRNA

The mechanism of splicing of the early and late cytoplasmic species of SV40 mRNA has been studied using a technique which first involves the isolation of specific regions of the viral genome. Restriction enzyme cleavage of SV40 DNA generates specific DNA fragments which can be fractionated using column chromatography. These fragments are then annealed to viral mRNA and the reaction products are fractionated by velocity sedimentation. The DNA fragment which is annealed to the viral mRNA is used as a primer for the enzyme, reverse transcriptase, and a complementary DNA copy of the mRNA is made. The sequence and structure of the cDNA has been compared with the known nucleotide sequence of SV40. This procedure has been used to characterize the late mRNA which codes for the major structural viral coat protein and has provided direct physical evidence for two early mRNA species. The regions of the DNA that have deleted in both species of early mRNAs have been determined. Also, the nucleotide sequences across these spliced regions have been determined and correlated with the mRNA coding for the large T and small t antigens. These two mRNA species were determined to have the same 5' termini which would suggest two levels for control of genetic expression. One would be the regulation of initiation of transcription at a common promoter; the other would involve post-transcriptional splicing.

To further characterize the mechanism of transcription of early SV40 RNA, nascent RNA chains that are attached to template DNA molecules are being isolated and characterized by the same methodology employed to study the processed cytoplasmic species of early viral RNA. Transcription complexes have been isolated from infected cells which continue viral mRNA synthesized in vitro. The structure of these nascent chains gives insight into the initial transcription products and the mechanisms that operate to regulate transcription. We are investigating differences between RNA's formed in vivo and in vitro. The structure of nascent chains will provide precise information about the primary products of transcription. We are also studying the use of isolated nuclei for the study of the regulation of transcription. Isolated nuclei are able to sustain in vitro synthesis of viral RNA. In this case, the SV40 template in the cell nucleus is present as a nucleoprotein complex containing the five histones. This is in contrast to in vitro synthesis of nascent RNA chains described above which uses a less well defined transcriptional complex.

These nascent RNA molecules will be probed with specific SV40 DNA fragments to determine their structure. In order to acquire a library of these specific fragments, recombinant technology has been employed. Cloning of SV40 DNA fragments using the plasmid pBR322 and E. coli should provide sufficient amounts of specific DNA fragments which can be used to probe nascent RNA molecules and determine the mechanisms operating during regulation of transcription.

Since sufficient amounts of specific SV40 DNA fragments can be obtained from cloning, we will be able to study how synthesis of specific RNA sequences are affected by mutations within the genome. Further characterization of RNA species obtained following Proflavin treatment, which blocks RNA processing, will aid our understanding of the mechanism that operated to regulate transcription. (Thompson, Bina-Stein, Salzman)

Localization of RNA Polymerase Promoters on SV40 DNA

Transcripts of SV40 DNA synthesized by Escherichia coli RNA polymerase have been characterized. This model system has been used for the development of new methods applicable to the analysis of the mechanisms involved in the synthesis of the viral messenger RNAs in SV40-infected cells. It has been previously shown that E. coli RNA polymerase recognizes specific initiation sites on SV40 DNA. Except for one of them, determination of the location of these sites on the SV40 DNA map is only approximate. No specific termination site for transcripts has been identified, and consequently RNA polymerase generates a heterogeneous population of molecules. The size of some of these RNA molecules is several times the length of the viral genome.

We have shown that, after binding of the E. coli RNA polymerase to SV40 DNA, it was possible to cleave such transcriptional complexes with "single-cut" restriction endonucleases (Bam H_T, Eco R_T, Hpa II). The addition of ribonucleotide triphosphates to these linearized complexes leads to the synthesis of defined species of RNA which can be analyzed by electrophoresis. The determination of the size of each of these RNAs together with the assignment of the DNA strand on which they are transcribed will allow the precise mapping of the various RNA promoters (Reuveni, Lavialle, Salzman).

Transcriptional Properties of SV40 Nucleoprotein Cores

Simian Virus 40 (SV40) provides an excellent model system for investigating the process of transcriptional regulation in eukaryotic cells. Many structural aspects of viral transcriptional complexes and mRNA molecules have been determined. In addition, the entire nucleotide base sequence of SV40 DNA has been determined. Similar to most eukaryotic chromatin, during the SV40 infection cycle, cellular histones H_2A, H_2B_1, H_3 and H_4 are bound to the viral DNA. Late in the infection cycle, histone H_1 also becomes associated with the bulk of the viral chromatin in a stable nucleoprotein complex. Just prior to encapsidation, the SV40 nucleoprotein complex undergoes a redistribution of proteins and histone H_1 is replaced by viral proteins. Since binding of H_1 on chromatin may suppress transcription of DNA sequences, we were interested

in determining if the viral proteins also acted to modify or regulate the process of RNA synthesis. Such regulatory properties could be important in a clearer understanding of the process of "early" mRNA synthesis during infection of the host cell. In view of the close similarity between this SV40 DNA-histone-viral protein nucleoprotein complex and cell chromatin structure, such studies would also be of interest in a more complete understanding of the general problem of transcriptional regulation in eukaryotic cells.

A nucleoprotein complex can be isolated from purified SV40 virions under very mild, physiological conditions. These nucleoprotein cores are potentially active transcriptional complexes. These core complexes do not contain endogenous RNA polymerase activity; however, 95-100% are able to form active transcriptional complexes. The transcriptional activity of those complexes is extremely high and is similar to the activity obtained with purified SV40 Form 1 DNA. This observation contrasts with published data, in which the level of transcription of nucleoprotein complexes (SV40 DNA-Histone) is generally less than 20% of the activity of deproteinized SV40 DNA. The results suggest that the viral proteins may play a role in the "activation" of the nucleohistone complex. Analysis of the composition and structure of the SV40 nucleoprotein core are being carried out and should allow an understanding of the unexpectedly high efficiency of the SV40 core as a template for RNA synthesis. (Brady, Lavialle, Salzman)

Publications

Chiu, N., Radonovich, M., Thoren, M., and Salzman, N. P., Selective degradation of newly synthesized non-messenger SV40 transcripts. J. Virol.,28: 590-599, 1978.

Seidman, M., Garon, C., and Salzman, N. P. The relationship of SV40 replicating chromosomes to two forms of the non-replicating SV40 chromosome. Nucl. Acids Res. 5: 2877-2893, 1978.

Panel V--Virus Task Force. N. P. Salzman, Chairman. NIAID Task Force Report, DHEW PUblication No. (NIH)79-1835

Bina, M., Thompson, A., Thoren, M., and Salzman, N. P. (1979) Rapid sequence determination of the late SV40 16S mRNA leader using inhibitors of reverse transcriptase. Proc. Natl. Acad. Sci. 76: 731-735.

Birkenmeier, E., Chiu, N., Radonovich, M., May, E., and Salzman, N. P. Regulation of SV40 early and late gene transcription without viral DNA replication. J. Virol. 29: 983-989, 1979.

Seidman, M., and Salzman, N. P. Late replicative intermediates are accumulated during SV40 DNA replication in vivo and in vitro. J. Virol. 30: 600-609, 1979

Thompson, J. A., Radonovich, M., and Salzman, N. Characterization of the 5'-terminal structure of SV40 early mRNAs. J. Virol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00126-06 LBV

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Restriction Enzyme Analysis of Vaccinia Virus DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: Frank DeFilippes, Research Physicist LBV NIAID

COOPERATING UNITS (if any)

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Laboratory of Biology of Viruses

SECTION
Macromolecular Biology Section

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NIAID, NIH, Bethesda, Maryland 20205

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	1	.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of the project is to study the organization of genes in large DNA molecules obtained from animal viruses. The production and ordering of unique segments of vaccinia virus DNA provide a physical map of the genome. Physical maps have been determined for several different restriction enzymes. I am also working to improve the techniques for separating and isolating very large pieces of DNA which differ in size and base composition by relatively small amounts. In particular, I am trying to isolate the DNA segments which contain the origin(s) of replication of vaccinia DNA and to test for possible signal elements in the DNA without prior knowledge of the nucleotide sequence.

PHS-6040
(Rev. 10-76)

Project Description

Vaccinia DNA has covalently-linked-complementary (CLC) ends which define each end of the physical map of the genome. These CLC end segments can be identified in each set of segments that are produced by a particular restriction enzyme. The set of segments is alkali denatured, neutralized and passed through a BND-cellulose column from which only the CLC segments elute as duplex chains. The end segments have been identified for several restriction enzymes such as Sal I, Hind III, Kpn I and Xho I. For Sal I, the end segments are identical and small with a mass of 2.1×10^6 daltons. For Xho they are larger (both 3.9×10^6) and for Hind III they are larger still (17.6 and 13.5×10^6).

The restriction enzymes listed above as well as several others are used to digest vaccinia DNA to obtain unique DNA segments which are separated by agarose electrophoresis. To obtain a physical map, segments produced by one enzyme are isolated from agarose and digested by a second enzyme to find overlapping regions. The end segments described here serve as reference points from which the maps are continued. The Sal I and Hind III map of vaccinia strain WR are now almost completely known. Kpn and Xho maps are also near completion.

A particular method which has helped to generate reproducible partial DNA segments involves restriction enzyme digestion in the presence of actinomycin D. The original observation that the drug blocked complete digestion with Hind III has been extended to 3 other restriction enzymes, Sal I, Xho and Kpn. None of these digests produce as long a partial as the one half length vaccinia DNA molecule generated with Hind III but they do contain several smaller linked segments which help to establish the map.

Initial experiments designed to locate the origin of replication involve pulse labeling HeLa cells with ^{3}H-thymidine after infection with high multiplicities of vaccinia virus. A peak of synthetic activity occurs 2.5 hours after infection. From 2.5 to 3.5 hours after infection alkaline and neutral sucrose gradients of cytoplasmic (viral) DNA reveal a spectrum of molecular sizes with about 10 - 20% of the DNA in full length molecules. These DNA species will be examined in a Dintzis (Proc. Natl. Acad. Sci. 47: 247, 1961) type experiment.

Several experimental techniques have evolved during the year. The method of Vogelstein and Gillespie (Proc. Natl. Acad. Sci. 76: 615, 1979) for extracting DNA segments from agarose with glass powder has been modified so that it is now possible to routinely recover intact, 70% or more of µg amounts of large vaccinia DNA segments. Also, poly rC columns have been used to hybridize segments which contain short stretches of GC rich regions in duplex DNA. Using this column technique, we have found that the largest Sal I segment binds to the column. Since GC rich regions may provide a structural basis for a signal element, the Sal I segment will be digested with other enzymes to localize the GC region. Finally, we have started to use malachite green columns to fractionate sheared vaccinia DNA (6×10^6 daltons) according to base composition.

Those fragments with the least affinity for the column have the highest average GC content. They will be radiolabeled and hybridized to Sal I digests to identify those segments from which they originated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00127-12 LBV

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (30 characters or less)
Structure and Function of Genetic and Protein Components of Defective Parvoviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Other:	Mark Buller	Visiting Fellow	LBV NIAID
	John Janik	Research Associate	LBV NIAID
	Edwin Sebring	Research Chemist	LBV NIAID
	Frank Koczot	Research Biologist	LBV NIAID
	Claude Garon	Research Microbiologist	LBV NIAID
	James Garrison	Bio Lab Tech (Biochem)	LBV NIAID

COOPERATING UNITS (if any)

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Laboratory of Biology of Viruses

SECTION
Molecular Structure Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
	4.0	2.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Main objectives in studying these defective human parvoviruses (AAV) are to (i) define specific biochemical mechanisms used in synthesizing their DNA, RNA and proteins, (ii) identify and characterize the helper virus-mediated step(s) required for their replication and (iii) relate biochemical findings to normal cellular processes as well as to potentials for selective interference with both AAV and Helper virus (adenoviruses, herpesviruses) infection. Among the methods used are nucleic acid hybridizations, analytical and preparative sucrose and CsCl sedimentations, molecular cleavage with restriction nucleases, gel electrophoresis and electron microscopy.

PHS-6040
(Rev. 10-76)

Project Description:

(1) Most, if not all, of the enzymes involved in the replication of AAV DNA probably play corresponding roles in cellular DNA synthesis. Among these activities, the enzyme(s) responsible for processing AAV concatemeric intermediates is of particular interest because of its site-specific attack at the origin of DNA replication. We have purified and partially characterized two single strand-specific endonucleases from KB cells that are possible candidates for such a processing enzyme. Both of these KB cell enzymes (KB_1 and KB_2) have relatively high pH optima (pH 9.2 and 9.5) and specifically cleave single-stranded DNA. However, the KB_1 enzyme differs from the KB_2 enzyme with respect to isoelectric point (KB_1 = 10.3; KB_2 = 6.4), absolute requirement for Mg^{2+} (KB_2 enzyme can also utilize Mn^{2+}) and relative rates of hydrolysis with homopolymers (for KB_1: dG>dT>dA>dC; for KB_2: dA>dT>dC>dG). Differences in rates of hydrolysis among the homopolymers indicate some specificity for different nucleotide bonds. Both enzymes hydrolyze poly(dT) 7-8 times more rapidly than denatured viral or cellular DNA. In addition, we have currently identified in KB cells several other distinct endonucleases that hydrolyze poly(dT) more rapidly than denatured DNA. It is planned to continue the purification and characterization of these endonucleases as well as to attempt to identify their roles in the cells and, possibly, the viral DNA replication process.

(2) Human adenovirus (Ad) serotypes provide an early factor(s) that is necessary for adenovirus-associated virus (AAV) multiplication in human cell lines. However, little if any AAV production occurs in primary African green monkey kidney (AGMK) cells co-infected with AAV and a helper human Ad (non-permissive infection) unless cells are additionally infected with SV_{40} (permissive infection). To determine the basis of the host restriction of AAV replication in AGMK cells, AAV DNA, RNA and protein synthesis were analyzed under various conditions of infection. Hybridization reactions revealed no detectable AAV-specific DNA or RNA in infections with AAV alone or in combination with SV_{40}. In co-infections with AAV and Ad_5 or Ad_7, the synthesis of both AAV- and Ad-specific DNA and RNA occurred without a significant rise in titre of either virus. During non-permissive infection, however, AAV DNA synthesis was abnormal in that an expected accumulation of single-stranded progeny molecules was not observed. Finally, although intact 20S AAV transcripts were present in the cytoplasm of AGMK cells during non-permissive infection (in amounts ranging from 50 to 80% of that found during permissive infection), AAV-specific polypeptides were not demonstrable by polyacrylamide gel electrophoresis. Taken together, these experiments indicate that the host restriction of AAV replication in AGMK cells is exerted at the level of translation of the single AAV messenger RNA. In addition, it appears that one or more of the AAV polypeptides specified by this message is required for the production of single-stranded AAV progeny DNA. The fact that coinfection with a simian adenovirus, SV15, permits complete replication of both Ad and AAV (analogous to coinfection with SV_{40}) strongly suggests that an Ad(SV15) gene product also exerts a regulatory effect on expression of the AAV mRNA (as well as several late human Ad mRNAs) in AGMK cells. Isolation and characterization of this

gene product should be relevant to possible approaches for selectively interfering with virus infection.

(3) Further understanding of mechanisms involved in DNA replication was obtained by studying AAV DNA synthesis in cell-free extracts. Extracts of nuclei from KB cells doubly infected with adenovirus-associated virus type 2 (AAV) and adenovirus type 2 (Ad) were examined for their ability to synthesize various molecular forms of AAV DNA. It was found that replicating AAV DNA molecules elongate to yield genome length hairpins or linear, open-ended duplexes. Both plus and minus DNA strands serve as templates for this synthesis which apparently does not reinitiate new rounds in vitro. Replicating Ad DNA molecules also could be identified in these extracts, but incorporation of [^{3}H]TTP into Ad DNA was diminished 80-90% in coinfections with AAV as compared to nuclei extracts prepared from cells infected with Ad alone. These results confirm our previous in vivo studies which indicated that a self-priming mechanism is involved in the replication of AAV DNA, studies that represented the first reported evidence for this mode of initiating DNA synthesis. Investigations with extracts will continue with the objective of identifying and characterizing enzymatic and regulatory components that participate in viral and cellular DNA replication.

Publications:

Buller, R. M. L., Straus, S. E. and Rose, J. A.: Mechanism of host restriction of adenovirus-associated virus replication in African green monkey kidney cells. J. Gen. Virol. 43: 663-672, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00128-12 LBV

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Properties of Adenovirus DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigators:	Claude F. Garon	Research Microbiologist	LBV NIAID
	James A. Rose	Medical Officer	LBV NIAID
Other:	Judy A. Sprague	Biologist	LBV NIAID
	James W. Garrison	Bio Lab Tech (Biochem)	LBV NIAID

COOPERATING UNITS (if any)

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	.4	.7

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major objective of these studies has been the application of physical and biochemical techniques to map and to define the structure of specific genetic regions in the genomes of both oncogenic and non-oncogenic human adenoviruses, with the ultimate goals of (1) relating these sequences to those biochemical activities which permit these viruses to influence or gain control of cellular functions (e.g., cell lysis and transformation) and (2) of defining basic mechanisms for regulation of genetic expression (e.g., initiation of DNA synthesis). A number of unique features have been described which may have implications in viral replication or carcinogenesis or both.

PHS-6040
(Rev. 10-76)

Project Description

The DNA of adenoviruses is normally isolated as a linear duplex molecule of discrete size using standard procedures of extraction with protealytic enzymes, phenol and SDS. Recently, it has been possible to demonstrate forms of DNA that are either circular or oligomeric by using procedures which do not involve protealytic enzymes. In these latter cases, the DNA molecules are always joined at their ends, and it appears that either end of a molecule may interact with the other end of the same molecule or with either end of another molecule. The circles and oligomers are resistant to treatment with 4M guanidinium chloride, 4M urea, formimide, sarkosyl or mercaptoethanol. Treatment with proteases, however, rapidly converts the circles and oligomers to linear double-stranded monomers, as does treatment with SDS. It had been previously proposed that there is a protein attached to each end of the DNA molecule and that this protein is responsible for the formation of the observed complexes. Subsequent studies have clearly demonstrated the presence of a 55,000 dalton protein very tightly linked to the DNA molecule. Although the function of the bound protein is unknown, it has been proposed that it may be involved in DNA replication by allowing initiation or completion of the 5' ends of the progeny strands. It has also been suggested that the protein may have a structural role by circularizing the DNA within the virus particle, may protect the DNA from exonuclease digestion or may act as an endonuclease in a hairpin model of DNA replication. Furthermore, it has been recently demonstrated that the efficiency of transfection with the Ad 5 DNA protein complex is about 100-fold higher than that of pronase-treated Ad 5 DNA.

Brown et al. (Journal of Virology 16, 366) first demonstrated that these DNA-protein complexes did not migrate into agarose gels during electrophoresis. This observation provided the basis for a useful means for detecting not only the presence or absence of terminal protein components but the identification and purification of terminal DNA sequences as well. We have fractionated the Ad 2 DNA-protein complex on hydroyapatite columnes and analyzed the DNA eluate by agarose gel electrophoresis. Although the initial DNA-protein complexed did not migrate into agarose gels, >75% of the initial DNA molecules in the DNA eluate were now able to enter the gels in spite of the absence of protease or SDS treatment. When the protein in the DNA eluate was labeled in vitro with ^{125}I, protein was not seen associated with the DNA that migrated into the gels. When the DNA eluate was examined in the electron microscope, DNA was found to be essentially linear. The re-addition of a DNA-free protein fraction to the DNA eluate produced circularization of about 20% of these linear molecules. We conclude that circularization of the adenovirus DNA-protein complex as well as its inability to migrate into agarose gels is likely due to an aggregate of a virion component(s) in addition to the covalently-attached 55K protein previously described.

Significant quantitative differences were noted when Ad 18, a highly oncogenic serotype, was extracted with 4M guanidinium chloride and the DNA-protein complexes assayed in similar fashion. Purified virions of adenovirus serotype 2 and 18 were applied to gradients

containing guanidinium, peak fractions were pooled and the DNA-protein complexes dialyzed and compared. 35-55% of the molecules from each serotype appeared circular in the electron microscope. While the DNA-protein complexes from serotype 2 were effectively bound to glass fiber filters (99%) under the conditions employed, over 45% of the guanidinium purified material isolated from serotype 18 passed unimpeded through the filter under identical conditions. Of some interest was the fact that nearly all molecules in this fraction were circular. Bound material could be effectively released from filters with 1% SDS or pronase digestion. DNA molecules from either serotype when extracted with proteolytic enzymes did not bind to filters. Approximately half of the guanidinium purified material from serotype 18 was shown to enter agarose gels upon electrophoresis. The structural implications of this population of molecules which apparently does not bind to glass fiber filters and which easily penetrates agarose gels, yet retains the ability to circularize, is of considerable biological interest.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00156-04 LBV

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Structural Characterization of DNA Virus Genomes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: Claude F. Garon, Research Microbiologist LBV NIAID

COOPERATING UNITS (if any)

Dr. J. W. Burnett, University of Maryland Hospital, Baltimore, Maryland
Dr. H. B. Bradford, Louisiana Health and Human Resources Administration
New Orleans, Louisiana

LAB/BRANCH

Laboratory of Biology of Viruses

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Macromolecular Biology Section

INSTITUTE AND LOCATION

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	0.2	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Molluscum contagiosum virions were isolated from clinically typical skin lesions and the DNA extracted and characterized using techniques of electron microscopy and agarose gel electrophoresis. The structural characteristics determined in these studies tend to place the MCV genome among other members of the poxvirus group in terms of genome molecular weight and organization. The apparent denaturability of the MCV genome into a continuous, single-stranded circle would point to the presence of terminal cross-links in the DNA molecule. So far, this structural arrangement appears to be unique to members of the poxvirus group. The objective of these studies has been to define structural features of these molecules and to relate them to the biochemical events involved in the replication and growth of this virus in the cells they infect.

Project Description

Molluscum contagiosum virus (MCV) is a member of the mammalian poxvirus subgroup which causes a benign tumor of the skin in humans. The disease is most common in children. Cases appear to occur world-wide and are often associated with poverty, overcrowding and poor hygiene. Molluscum contagiosum virions were isolated from clinically typical skin lesions and the DNA extracted and characterized using techniques of electron microscopy and agarose gel electrophoresis. In some experiments, a comparison of the structural features of the MCV genome and other members of the poxvirus group was made.

Extracts from molluscum contagiosum lesions when stained with sodium phosphatungstate and examined in the electron microscope appeared to contain many structurally mature virus particles of both C and M forms. However, the virus has not been successfully propagated in the laboratory. Molecular information about this virus has been limited to description of size, shape and composition and has been difficult to obtain. The structural characteristics determined in our initial studies (Virology 81: 247) tend to place the MCV genome among other members of the poxvirus group in terms of molecular weight and organization. The average molecular weight of MCV-DNA was calculated to be 118 X 10^6 and appeared to be extremely sensitive to both mechanical shear and nuclease damage. Single-stranded circles measuring twice the length of linear molecules were observed following high levels of denaturation. This now appears to be a generalized feature of all poxvirus DNAs so far examined. The biological function, if any, of the unique terminal-cross-links is unknown. Also of special interest was the fact that the most easily denaturable regions of the MCV genome (and presumably that of the highest AT content) appeared to be the end 19% of the DNA molecule. That the MCV genome differ markedly from that of vaccinia is clear from the visual denaturation profiles and from the restriction endonuclease cleavage patterns obtained in our initial studies. Further differences were noted in the extractability of the various poxvirus genomes. Procedures used for successfully extracting and purifying the DNA molecules from one viral genome proved completely unsuitable for the other.

Previously, little was known about whether most isolates belonged to the same or different MCV strains on whether a given strain was always isolated from similar clinical sources. A survey of the restriction endonuclease fragment patterns of several independent MCV isolates was initiated. Lesions obtained from individual patients were never pooled but rather purified, extracted, and assayed as a single isolate. Initially, eleven such isolates were processed. In these studies, lesions isolated from various sites on an individual patient appeared to contain virus whose DNA showed identical gel patterns. Furthermore, virus was isolated from the lesions of patients' relatives which, when assayed by the above restriction endonuclease procedure, showed gel patterns identical to those of the patients themselves. Interestingly, only 3 characteristically frequent patterns were observed among eleven independent isolates. Restriction endonuclease digestion produced 11 to 20 fragments ranging in size from 4 X 10^5 to 31 X 10^6 daltons depending on the virus isolate.

Mixing experiments showed comigration of some fragments in all patterns. Although comigration of DNA fragments does not necessarily mean base sequence identity, extensive comigration would imply significant genetic or organization similarity among the viral genomes. Direct comparisons of these gel purified fragments by hybridization or heteroduplex analysis have not been attempted due to limits in the quantity of viral DNA obtainable from each MCU isolate.

Although we have not collected a large enought body of data to make any reasonable assessment of the relationship between the clinical characteristics of the disease and the restriction endonuclease cleavage patterns, it may be possible one the data are accumulated, to relate these observations and to develop a useful molecular epidemiological scheme. Such a scheme would make possible rapid identification and classification of MCV isolates without the necessity of propagating the virus in the laboratory. Furthermore, since the degree of sensitivity of such a system is limited only by the number of cleavage sites recognized by a given restriction enzyme, other available enzymes may easily be substituted.

We propose to continue to define structural features of these poxvirus DNA molecules with the aim of (1) comparing genetic variation among members of this group, and of (2) relating these features to those biochemical events which are involved in the replication of these agents.

LABORATORY OF CLINICAL INVESTIGATION
1979 Annual Report
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Summary of Program
Laboratory of Clinical Investigation
October 1, 1978 - September 30, 1979

Michael M. Frank, M.D., Chief of Laboratory
and Clinical Director, NIAID

Introduction

This has been a highly rewarding year for the Laboratory of Clinical Investigation. It has been a year of continuing change and development of the new directions of the program. Basically, two years ago a decision was made to continue to have represented within the laboratory strong programs in infectious diseases, immunology and allergy. Several of the senior members of the staff left for other excellent appointments and an opportunity was had to change the directions of the laboratory at a time when there was no possibility for further growth in positions or space. A decision was made to allow for modest increases in the size of the commitment toward cellular immunology under Dr. Fauci, toward complement research under Dr. Frank, toward allergy research under Dr. Kaliner and toward research in the phagocytic cell in host defense under Dr. Gallin. Our commitment to mycology continues to be evident and strong and our commitment to virology was reaffirmed with the appointment of a new leader of the virology program.

Dr. Fauci had the opportunity to recruit a second senior individual in cellular immunology, Dr. Barton Haynes, who joined the senior staff. The Clinical Immunology Section recruited one of its former Clinical Associates, Dr. Steven Hosea, to join the staff as an independent investigator developing the area of the pathophysiology of bacterial infection. Dr. Kaliner assumed the position of Chief of the Allergy group with the departure of Dr. Allen Kaplan and was given a second position to recruit a second senior allergist. Dr. Dean Metcalf, one of our former Clinical Associates has been recruited and will be joining the laboratory at the end of theis fiscal year. Dr. Metcalf served 3 years as a Clinical Associate in the Laboratory of Clinical Investigation and then joined Dr. Frank Austin's department at Harvard where he continued to develop these investigative skills. He will be starting a program in food hypersensitivity. Thus, the various groups have continued to be rounded out in the face of very limited potential for growth.

The Clinical Immunology group now has extensive depth in the general area of complement protein interactions and contribution of complement to medical illness. The group has extended its depth considerably in the general area of immune complexes and the pathophysiology of immune complexes and has worked very closely with members of the Cancer Institute interested in these same problems. The cellular immunology program has extended under Dr. Fauci and Dr. Haynes and is deeply involved in lymphocyte function in autoimmune diseases. We continue to study the pathophysiologic responses of the phagocytic cell and continue to develop our capabilities in bacterial infection, various aspects of mycotic infection.

During this year Dr. Raphael Dolin left to assume the position of Chief of the Infectious Diseases at the University of Vermont. After an extensive search Dr. Stephen Straus was added to the program to continue and develop our strength in virologic investigation. Dr. Straus comes to us from Washington University in St. Louis where he received extensive training in infectious diseases. Before that he was trained in basic virology in the NIAID Laboratory of the Biology of Viruses. As discussed below he will extend and develop our expertise in the the use of antiviral agents and in the pathophysiology of viral infection.

Dr. Charles Kirkpatrick has continued his studies of mucocutaneous candidiasis and of delayed hypersensitivity defects in a wide variety of clinical diseases. Dr. Kirkpatrick has accepted a position as Professor of Medicine in Denver and will be leaving the Institute at the end of this fiscal year. Nevertheless, it is hoped that our expertise in cellular immunology will be maintained through Dr. Fauci's efforts in this area. Dr. Kirkpatrick has made enormous strides in the development of rational treatment for patients with mucocutaneous candidiasis and in our understanding of the pathophysiologic basis of this disease. It can safely be said that his efforts have been responsible for most of the available therapies which are now in use in treatment of this disease. Moreover, his contributions to the development of our understanding of transfer factor have been considerable and it is expected that he will be able to continue his efforts to understand the structure of transfer factor and its mechanism of action in his new position in Denver.

Research Accomplishments

CLINICAL VIROLOGY SECTION

Dr. Straus is continuing the ongoing effort of the viral disease section in exploring the pathogenesis and natural history of herpes virus infections in man. Moreover studies of infections mononucleosis have been extended during the year and a number of effects of the causative agent the EB virus, upon the target B lymphocyte have been examined. Moreover effects of EB virus on T cell responses and on helper and supressor T cell function have been examined. The group continued and confirmed earlier observations on the usefulness of adenine aranoside in therapy of serious herpes virus infections. An enzyme linked immunoadsorbant assay was also developed for detecting influenza A hemagglutinin, candida cell wall mannan, human adenoviruses, herpes simplex virus and antibodies to pneumococci. These important diagnostic advances will make rapid diagnosis and patient evaluation much more practicable as they are further developed. Dr. Straus initiated studies of enteral adenovirus infection as a cause of gastroenteritis in children. These viruses are very difficult to grow and a collaborative study has been initiated to try several methods of approach to the culture of these viruses and to the study of their pathophysiologic effects. It is hoped to add studies of this viral group to the ongoing studies in NIAID of the causes of viral gastroenteritis including the addition of viral challenges of normal volunteers to define the pathophysiology of these infections.

CLINICAL MYCOLOGY SECTION

Dr. Bennett continued his investigations of host defense reactions to Cryptococcus neoformans. The immunodominant groups of the type A form of the Cryptococcus were studied in an attempt to determine the precise mechanisms of antigenicity. In this Cryptococcal type, defined originally by Drs. Kwon-Chung and Bennett, the immunodominant group was found to be 1 3 mannan as a backbone with Oacetyl groups and to a variable extent the glucuronyl side chain. An ELISA assay was also developed for the detection of Aspergillus fumigatus. Under Dr. Kwon-Chung efforts were extended to understand the various pathways utilized in Cryptococcus neoformans and C. bacillisporus. Also the nuclear states of basidiospores in the sexual development of C. neoformans were studied as were the mating types among the various groups which have been observed in this laboratory. The observation that Cryptococci can be divided into distinct species is considered of major importance in understanding the biology of this organism and perhaps in explaining some aspects of the host defense mechanisms engaged by these organisms.

CLINICAL PHYSIOLOGY SECTION

Dr. Fauci's unit has continued to explore the precise mechanisms and immunoregulatory events associated with triggering of B lymphocytes following nonspecific and antigen induced stimulation. Functionally distinct as well as overlapping immunoregulatory lymphocyte subsets have been characterized. The precise abnormalities of immunologic reactivity have been determined in several autoimmune diseases such as systemic lupus erythematosus, Sjogren's Syndrome, infectious mononucleosis, sarcoidosis, and tuberculosis. Dr. Fauci's group has succeeded in producing an anti-ideotypic antibody which specifically blocked the function of human B cells bearing the corresponding antibody ideotype. Hybridoma lymphocyte cell lines were established which are secreting antibody which binds specifically to functionally distinct subsets of human blood lymphocytes. The availability of such antibodies should prove of major importance in further exploration of immunologically mediated effects in humans. Countercurrent centrifugation techniques were adapted from those previously described and which result in 95% purity and yield of monocytes from human blood. Again, the availability of such highly purified cells should greatly ease further developmental work in cellular immunology. Modulation of lymphocyte subsets by a number of clinically relavant pharmacologic agents has been further defined. Clinical therapeutic studies in this group have yielded highly favorable results in the approach to the treatment of systemic necrotizing vasculitis. A number of systemic necrotizing vasculities have yielded to therapy with cyclophosphamide or cyclophosphamide and glucocorticoids.

CLINICAL IMMUNOLOGY SECTION

This Section has continued its studies in three major areas, the role of complement in the production of disease, the structure and function of immune complexes in disease and the role of the reticuloendothelial system in the development of autoimmune illness and in host

defense mechanisms. In the first of these areas great strides have been made in the purification of large quantities of complement components which are available for study in patients. This has required an enormous effort in terms of protein purification and new methodologies have been developed for the purification and separation of functionally active components. Virtually all of the components of the classical pathway have now been purified and are now available for study as are certain of the regulatory proteins of the complement system. A new method has been developed for the evaluation of the opsonic fragment of C3 and it has been possible to study the degradation of C3 on a molecular basis in precise quantitative detail. This complement protein is of major importance in bacterial destruction and further understanding of its mechanisms of action is of great importance. Moreover, methods have been developed for analyzing these mechanisms of degradation in normal sera. Studies of complement receptor function and Fc immunoglobulin receptor function have continued to provide great insight into normal pathophysiologic mechanisms and disease states. Thus, for the first time a receptor for C5 has been identified unequivocally on polymorphonuclear neutrophils and the binding of C5 to C3 has been studied extensively. The role of phagocytic cells in the degradation of and decay of C3 on opsonized particles has also been extensively explored. Tests of in vivo reticulo-endothelial function in man have been developed which are now coming into wide use around the world. It has been demonstrated for the first time that certain kinds of autoimmune diseases associated with the presence of circulating immune complexes are also associated with defects in the function of the reticuloendothelial system, and other autoimmune diseases associated with circulating immune complexes are not. In general the autoimmune diseases associated with clearance defects are those which have as part of the clinical spectrum tissue deposition of immune complexes and tissue damage. Thus in general the most severe autoimmune diseases are associated with defects in immune clearance. Extensive studies have been performed in lupus erythematosus, mixed connective tissue disease, rheumatoid arthritis, mixed cryoglobulinemia, Sjogren's Syndrome, primary billary cirrhosis and chronic active hepatitis, etc. Defects in immune clearance which are specific for various types of receptors on phagocytic cells have been demonstrated as well. Importantly, the standard test for RES function in man, that which measures clearance of aggregated human serum albumin, has been shown to be useless in these evaluations. A large population of patients with Hereditary Angioedema have continued to be followed and evaluated. New therapy is being developed as is further understanding of the pathophysiology of this interesting "experiment of nature".

CLINICAL ALLERGY AND HYPERSENSITIVITY SECTION

Dr. Kirkpatrick's laboratory has continued to study the biologic basis of cellular immunity. Continued studies of a large group of patients with mucocutaneous candidiasis has shown a proportion of

these patients have an abnormal ratio of helper T to suppressor T lymphocytes, suggesting a problem in the regulation of immunologic function. Dr. Kirkpatrick's group has isolated a new lymphokine termed E-RAF which appears to induce the maturation of null cells into E-rosette forming T cells. He has shown that there are several products which can be elaborated from lymphocytes which produces these effects and that certain kinds of patients with immunologic deficiency do not produce this factor. In Dr. Kirkpatrick's laboratory several controlled clinical trials of new agents for use in treatment of mucocutaneous candidiasis have been conducted including a control trial that has shown that clotrimazole troches are effective therapy for oral candidiasis. It has also been shown that transfer factor may induce more prolonged remissions from candidiasis in patients who receive this therapeutic agent.

ALLERGIC DISEASES SECTION

The Allergic Disease Section has continued to widen its investigations of the basic immunologic and physiologic events in immediate hypersensitivity reactions and in the pathophysiologic processes which take place in patients with a variety of allergic conditions. A number of areas are now being studied in detail and although the work is only in its infancy a wide variety of important leads have already been unearthed. One project involves characterization of mucous secretion and identification of the factors which control mucous secretion. This is quite important because excessive mucous secretion in such diverse diseases as asthma and cystic fibrosis play a major role in the pathology of the disease. Methods have been developed to study mucous secretion and the mucous products have now been identified and radiolabelled. The effect of drugs on mucous secretion is now under study. The site and control of human lung parenchyma as opposed to airway prostaglandin production has also been partially identified and the factors generated during the anaphylactic response which cause prostaglandin synthesis have been partially isolated. In another area of investigation the histologic responses to the injection of mast cell granules have been characterized both in rat and monkey skin and those factors responsible for eliciting the inflammatory response have been partially isolated and characterized.

Detailed studies have been performed on the autonomic nervous system in patients with asthma and allergic rhinitis and a number of important points have been discovered. Among these is that patients with asthma and allergic rhinitis have significant impairment of beta adrenergic responsiveness and increased responsiveness to cholinergic stimuli. Patients with allergic rhinitis have normal response to alpha adrenergic stimulation while asthmatics have augmented response to alpha-adrenergic stimulation. These stimuli control the responsiveness and state of dilatation of the airways of the human lung and these observations are of direct relevance in our attempts to understand asthma.

BACTERIAL DISEASES SECTION

Dr. John Gallin's laboratory has continued to study various aspects of phagocytic cell function. Mechanisms of leukocyte activation by chemotactic factors have been studied using electrophysiologic techniques as well as techniques which probe cell surface charge and ultrastructure. Dr. Gallin's group has shown that secretion of specific granules which may accompany chemotaxis is associated with increased cell adhesiveness and increased availability of chemoattractant receptors. Vigorous exocytosis is associated with depressed chemotaxsis, decreased availability of chemoattractant receptors, hydrolysis of the chemoattractant by secreted products and markedly increased cell adherence and aggregation. Human pyrogen has been shown to be a potent stimulator of neutrophil exocytosis and causes activation of the hexose monophosphateshunt. Dr. Gallin's laboratory had previously shown that neutrophils can be divided into two populations, those with large numbers of Fc receptors and those with small numbers of Fc receptors. Recent work has shown that both sets of neutrophils have equal C3b receptor activity and that neither has a demonstrable C3d receptor. Biological differences however between the two sets of granulcytes have been clearly indicated. Thus, in vivo injection of endotoxin into patients or the hemodialysis of patients leads to loss from the circulation of those neutrophils with demonstrable Fc receptors and a marked increase in the percentage of granulocytes with no demonstrable Fc receptors.

BIOLOGIC STRUCTURE SECTION

Dr. Allen Rosenthal left the Laboratory of Clinical Investigation to assume the position of Director of Immunology of the Merck Sharpe and Dohme Company one year ago. At that time he had a number of fellows and a number of important research projects which are of interest to the Institute. During this year those projects were partially or entirely completed.

It was shown that antiinsulin antibodies of exquisite antigenic specificity can be prepared and identified. Moreover, it was shown that the cellular immune response to the insulin antigen does not necessarily recognize the same antigenic groupings as the humoral response. Precise fine structural analysis of the actual amino acids recognized during the cellular response to insulin has been defined and initial experiments have begun on determining the role of histocompatibility groups on antigenic recognition of portions of the insulin molecule. This is a far ranging project which may ultimately give us a great deal of information about the biological basis of insulin allergy. Dr. Rosenthal intends to continue these investigations at the Merck Sharpe & Dohme Company.

In all, this has been an impressive year in the Laboratory of Clinical Investigation. Both clinically and in terms of basic science great strides have been made. Our research groups find themselves with many more problems than we can possibly find time to investigate and we all look forward to future developments with great anticipation.

CLINICAL PARASITOLOGY SECTION

The parasitic disease group has continued to extend its basic studies of Chagas' disease, schistosome and filariasis. The association of absence of HLA tissue type-A1 with chronic Chagas' disease was not confirmed by an expanded study of cases, but DR (B cell) typing will still be done on frozen cells. The diversity of HLA haplotypes encountered in this Brazilian patient population corroborates what anthropologists have said of this population - namely, extreme genetic mixing. Further study of different antigenic fractions of schistosome worms has disclosed one fraction to which antibody levels in patients correlate quite well with numbers of eggs being excreted, or intensity of infection. Evidence for serum inhibitory factors and for suppressor cells has been found in filariasis patients characterized by unresponsiveness to filarial antigens. The role of immune complexes in filariasis patients is also being studied. Serum and cell factors were also found that contributed to antigen unresponsiveness in patients with chronic schistosomiasis.

Honors and Awards

This has been a year in which the efforts of members of the Laboratory have been well recognized. Members of the Laboratory have been elected to many learned societies and now serve on the Editorial Board of many major journals.

Specifically, Dr. Anthony Fauci was elected President of the American Federation for Clinical Research and received the Public Health Service Meritorious Service Award. Dr. Fauci joins Dr. Frank as a member of the Association of American Physicians. Dr. Frank was invited to give the plenary lecture at the Japanese Rheumatism Association Meeting in Japan in June of 1979 and Dr. Fauci gave the Stanislaus Jaros Memorial Lecture to the American Association for Clinical Immunology and Allergy in 1978. Dr. Frank was invited to speak at the plenary session of the Infectious Diseases Society Meeting in 1979. Dr. Michael Kaliner was elected to the American Society for Clinical Investigation bringing the number of members of that Society within the LCI to five. Dr. Jack Bennett was elected to the Council of the Infectious Diseases Society and Dr. Kirkpatrick was appointed to the Education Committee of the American Academy of Allergy. The American Academy of Allergy also appointed Dr. Fauci to the Postgraduate Education Committee. In addition, Dr. Gallin was appointed to the Program Committee of the American Association of Immunologists. Dr. Kwon-Chung was elected Chairperson of the Medical Mycology Division of the American Society for Microbiology and was also selected Society Lecturer for the British Mycopathological Society at the Annual Meeting in Birmingham, England.

The Laboratory of Clinical Investigation is also well represented on Editorial Boards of major journals. Dr. Frank and Dr. Fauci are now both serving on the Editorial Board of the Journal of Clinical Investigation. Dr. Fauci serves as Section Head of the Editorial Board in Clinical Immunology of the Journal of Immunology and Dr. Gallin is a member of the Editorial Board. Dr. Fauci serves as a Section Head of the Editorial Board in Allergy and Immunology of the American Journal of Medicine. Dr. Frank serves on the Editorial Board of Blood and on the Editorial Board of the new Journal of Infectious Disease Reviews. Dr. Bennett serves on the Editorial Board of the Journal of Infectious Disease and the Journal, Antimicrobial agents and chemotherapy and on the Editorial Board of the Journal of Clinical Microbiology as well. Dr. Kirkpatrick serves on Editorial Boards of three journals, Thymus, The Journal of Allergy and Clinical Immunology, and the Journal of Cutaneous Pathology. Dr. Gallin serves on the Editorial Board of the Journal of Immunology and also of the new journal, Inflammation. Dr. Kaliner serves on the Editorial Board of the Journal of Allergy and Clinical Immunology. Dr. Fauci serves on the Editorial Board of the Journal of Immunopharmacology and on the Editorial Board of the American College of Physicians, Medical Knowledge. Self-Assessment, Audiocassett Program. Dr. Kwon-Chung serves on the Editorial Board of the Journal, Sabauraudia. Thus, many major journals in modern clinical immunology in infectious disease and clinical research are represented by members of the Laboratory.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00043-14 LCI

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Immunology and Chemotherapy of Systemic Mycoses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.E. Bennett	Head, Clinical Mycology Section	LCI NIAID
OTHER:	A.D. Hernandez	Guest Worker	" "
	D.K. Henderson	" "	" "

COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Clinical Investigation

SECTION

Clinical Mycology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3 11/12	3 11/12	2.0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The immunodominant groups on type A Cryptococcus neoformans were found to be the α1,3 mannan backbone, particularly the O-acetyl groups and, to a variable extent, the β-glucuronyl side chain.

Radioimmunoassay and ELISA have been developed to detect a specific neutral polysaccharide of Aspergillus fumigatus.

PHS-6040
(Rev. 10-76)

Project Description:

The general goals of the unit have been to study host defense in systemic mycoses and to improve diagnosis and treatment of these disorders.

Objectives:

1) Determine the role which antibody to C. neoformans plays in host defense; i.e., the cause and significance of absent antibody formation in most patients with the disease.

2) Characterize the immunodominant groups which contribute to serotype specificity in C. neoformans polysaccharides.

3) Purify polysaccharides of Aspergillus fumigatus, devise assays for these polysaccharides and determine whether these antigens appear in body fluids during infection.

Methodology, Results and Significance:

1) Hapten inhibition radioimmunoassay has been used to explore further the immunodominant positions on C. neoformans polysaccharides. Although individual sera do vary, antibody binding sites on type D polysaccharide include the α1,3 mannan backbone, particularly the O-acetyl groups there, and, for occasional sera, the glucuronyl side chain. On type A, β-glucuronyl mannan is one determinant. None of these determinants has proven immunodominant in type C; and it seems likely that larger, more complex structures are immunodominant. Lack of O-acetyl groups and extensive substitution of the α1,3 mannan backbone are the structural features of type C which account for these differences in immunodominance. (Bennett and Bhattacharjee)

2) Young healthy volunteers were found to have IgM and IgG antibody to type A C. neoformans polysaccharide. Antibody was significantly less common beyond 50 years of age, a time in life when cryptococcosis is more common. The majority of patients with cryptococcosis do not have anti-cryptococcal antibody during either illness or in later years. It remains to be determined whether disease produces tolerance or inability to respond to the antigenic challenge reflects a predisposing cause of infection. (Bennett and Henderson)

3) A neutral polysaccharide of Aspergillus fumigatus was tyraminylated and labeled with ^{125}I. From 40-50% of the radioactivity was precipitable with rabbit antisera. Although the polysaccharide has a fairly homogeneous pattern on Sephadex chromatography (mol. wt. 36,000 daltons), the portion of the polysaccharide not binding to antibody should be able to be removed by affinity chromatography. The final material can be used for structural analysis and to search for antigenemia in aspergillosis.

The partially purified polysaccharide can be detected by ELISA down to 100 ng/ml. This assay also will be used to search for antigenemia in

aspergillosis. (Bennett and Kim)

4) Radioimmunoassay has found antibody against C. albicans mannan in both normal volunteers and immunosuppressed patients, with no substantial differences between these groups. Small numbers of patients with fatal disseminated candidiasis have had antibody titers similar to the other two groups.

Publications:

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2. Feldman, L., Lamson, M., Gallelli, J.F. and Bennett, J.E.: Surveillance of nosocomial infections by antibiotic monitoring. J. Amer. Med. Assoc. 241:2806, 1979.

3. Bennett, J.E.: Diagnosis and management of candidemia in the immunosuppressed host. Scan. J. Infect. Dis. Supp. 16:83-86, 1978.

4. Mandell, G.M., Douglas, R.G., Bennett, J.E.: Principles and Practices of Infectious Disease. John Wiley and Sons, New York, N.Y., 1979.

5. Segal, E., Berg, R., Pizzo, P. and Bennett, J.E.: Detection of Candida antigen in the sera of patients with candidiasis by an ELISA inhibition technique. J. Clin. Microbiol. 10:116-118, 1979.

6. Bennett, J.E., Dismukes, W.E., Duma, R.J., et al: A collaborative study comparing amphotericin B alone or combined with flucytosine in the treatment of cryptococcal meningitis. N. Engl. J. Med. 301:126-131, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 Al 00045-11 LCI

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Studies on the Interaction of Antibody and Complement on the Production of Immune Damage

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Frank	Head, Clinical Immunology Section and Chief	NIAID,LCI
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	T. Gaither	Resident Biologist	NIAID,LCI
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	L. Renfer	Chemist	NIAID,LCI
	M. Santaella	Guest Researcher on IPA	NIAID,LCI
	H. Gresham	Medical Technologist	NIAID,LCI
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	S. Hosea	Fellow in Infectious Diseases	NIAID,LCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Clinical Investigation

SECTION
Clinical Immunology Section

INSTITUTE AND LOCATION
NIH, NIAID, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5 4/12	2 10/12	3 6/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Techniques have now been developed in the laboratory for the large-scale purification of C1q, C4, C3, 5, 6, 7, 8, 9, and the C56 complex. New assays have been developed for the C3b inactivator and for the protein β1H and the role of these proteins in C3b inactivation has been further explored. Studies are ongoing on the characteristics of this interaction which leads to the destruction of the opsonically active C3b site on erythrocyte surfaces. Studies have also been conducted on the interaction of complement with bacteria and considerable progress has been made in studies of the role of complement in producing the clinical symptoms of infectious disease.

PHS-6040
(Rev. 10-76)

Project Description:

Objective:

The objective of this program is the longterm study of the role of antibody and complement in the production of immune injury in vitro and in vivo. To this end the changes which were initiated last year in the Clinical Immunology Section have continued. A pronounced emphasis is continuing on protein purification and assay and on studies of protein interaction within the complement system with the goal of developing reagents suitable for pathophysiologic studies in patients.

Methods Employed:

The methods employed involved purification of complement components, their use in the sequential build-up of complement intermediates on cells, the identification of sera of patients with defined complement abnormalities so that these sera can be used as reagents, and the purification of immunoglobulins of various classes by standard techniques. Methods for titration of complement components have been developed in our laboratory, and these are in routine use. Methods for cultivation of various types of bacteria as well as for bactericidal assays have also been developed in the laboratory and methods for radiolabeling of bacteria. Disc gel polyacrylamide electrophoresis as well as electrofocusing techniques have been developed in the laboratory and methods have been developed to prepare proteins of very high purity. Also large batch methods have been developed for the first time for the purification of the various complement proteins. Methods have also been developed for the radiolabelling of bacteria with ^{125}I.

Major Findings:

Using methods initially developed for the purification of C3, C5, C6, and C7 from separate plasma pools we have consolidated the purification protocol to allow for the isolation of most of complement proteins in biologically active, homogenous form from one large pool of human plasma. The basis of success of this purification scheme has rested on the ability to obtain a large number of selective highly resolved chromatographic pools early in the purification protocol in which biological activity has been stabilized. We have found that the use of specific protease inhibitors and agents which block complement activation is requisite for recovery of biological activity of many of the complement proteins. We have demonstrated that in addition to the use of inhibitors in preservation of complement activity the ability to proceed rapidly through the first ion exchange steps yields improved recoveries of biologically active complement proteins. Using the biochemically homogeneous components we have begun to prepare monospecific antisera in goats and burros. These reagents are of indispensable value in clinical studies to determine antigenic levels in patients and to further study the interaction of the proteins and their biological activity.

This year has also seen the completion of a new assay system which quantitates the functional activities of the alternative pathway control proteins β1H and C3b inactivator. Homogeneous C3 preparations radiolabelled with ^{125}I iodine are used to prepare cellular intermediates in the complement sequence and the use of these intermediates has allowed us to clarify the functions of βIH, the C3b inactivator and a proteolytic serum enzyme involved in inactivation of the biologically important C3 fragment C3b. The assay is based on the release of radiolabelled C3c which occurs only after all of these proteins have interacted with C3b bound to the cell membrane. The C3b cleavage fragments formed during inactivation of cellbound C3b have been characterized by SDS polyacrylamide gel electrophoresis. This analysis verified the C3b molecule undergoes two separate cleavages, first by the C3b inactivator and β1H and, second, by the proteolytic enzyme. The assay is presently being applied to study β1H and C3b inactivator activities in the sera of patients with a variety of illnesses. Experiments are currently being designed to study the inactivation of C3b bound to the surface of various strains of bacteria. The goal is to determine if there is relationship between virulence of various bacterial strains and the susceptibility of the bacterial bound C3b to inactivation by the control proteins. Preliminary studies have been performed using S. typhosa 0901 to establish the optimal conditions for C3 inactivation and C3 binding and for inactivation of the bound C3b by purified control proteins. Studies of interaction of bacteria and complement have proceeded with the development of new techniques for radiolabelling of bacteria. These studies will be extended to consideration of the role of complement in the development of the clinical signs and symptoms associated with bacterial infection.

Significance to Biomedical Research

At present it is clear that complement activation plays an essential role in protecting the individual from infection with microorganisms. It is also clear that unregulated complement activation is a crucial component of autoimmune disease and regularly leads to tissue injury. Very little is known about how the complement proteins function in disease states however. The studies underway in our laboratory at present will make it possible to explore each of these questions in a systemic manner. They represent the overcoming of formidable methodologic barriers and are essential to further progress in theses areas.

Proposed Course:

We plan to extend these studies to obtain a further understanding of the role of the proteins in the regulation of complement function in normal physiologic situations and in disease states. We plan to examine the role of control proteins in infectious states and in control against bacterial infection. In addition, we plan to determine the role of complement activation fragments in the development of clinical signs and symptoms of bacterial disease. We plan to further purify complement proteins and components so that we can study their metabolism and interaction in a variety of disease states.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00046-11 LCI

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Pathogenesis of Delayed Hypersensitivity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Charles H. Kirkpatrick	Head, Clinical Allergy & Hypersensitivity Section
OTHER:	Eskild A. Petersen	Guest Worker
	A.I. Agbata	Visiting Fellow, NIAID, LCI
	M. Adedin	NICHD, LCI
	J.I. Gallin	NIAID, LCI
	L.E. Greenberg	Bio. Lab. (Micro., NIAID, LCI

COOPERATING UNITS (if any)

J. Cook & A. Lewis, OSD, NIAID, S. Shama, Dermatology Branch, NCI, S. Gupta, Sloan-Kettering Cancer Center, P.G. Sohlne, Medical College of WI, D.W. Alling, OSD, NIAID.

LAB/BRANCH

Laboratory of Clinical Investigation

SECTION

Clinical Allergy and Hypersensitivity Section

INSTITUTE AND LOCATION

NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4 9/12	2 9/12	1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The nature of the immunologic defect that predisposed certain patients to chronic and recurrent fungal infections such as mucocutaneous candidiasis has been further defined. Certain patients have abnormal ratios of Tμ to Tδ lymphocytes that are compatible with a problem with regulation of immunocyte function.

A new lymphokine, E-RAF, has been defined. It seems to induce maturation of "null" cells into E-rosette-forming T-cells. These observations indicate that "null" cells are not "null", but are pre-T-cells.

Two therapies for chronic mucocutaneous candidiasis have been studied. A controlled clinical trial has shown that clotrimazole troches are effective therapy for chronic oral candidiasis. Transfer factor from candida-sensitive donors may restore the cellular immune responses to candida to these patients and prolong drug-induced remissions.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives (sub-project A):

1) Characterization of cellular immune responses in immunologically deficient patients with chronic infectious diseases;

2) Studies of the pathogenesis of acquired and congenital immunologic deficiencies;

3) Studies of lymphokine production by cells from normal and immunodeficient subjects:

4) Studies of developmental immunology including studies of the immunologic activities of human cord-blood lymphocytes and maturation of cell-mediated immune systems in Syrian hamsters.

Methods Employed

The major portion of our studies of cellular immune deficiency have been conducted in patients with chronic mucocutaneous candidiasis. Approximately 60 patients with this syndrome have been evaluated. Evidence of immunologic deficiency has been found in 66% of the patients.

The general model for the pathogenesis of cellular immunodeficiency that we presented in 1971 still serves as valid outline in which to conduct studies of host-defenses. According to his model, patients with candidiasis developing during infancy or early childhood have a congenital basis for their immunologic deficits. Other patients are apparently normal until adult years when they develop candidiasis. In these patients, the immunologic abnormalities are probably acquired. This problem has been under study and measurements of immuno-regulatory activities in candidiasis patients and the subpopulations of T-lymphocytes have been measured.

In addition, the biological role of a lymphokine, E-rosette augmenting factor (E-RAF), that was discovered by Dr. Agbata in our laboratory has been under study in patients with immunodeficiency syndromes both before and after they were treated with immuno-reconstitutive measures.

The studies of development of immunocompetence by human and hamster lymphoid cells have continued.

The collaborative studies (with J.I. Gallin) on the relationships between cellular immunity and chemotaxis in patients with exceptional susceptibility to pyogenic and/or fungal infections have continued. The studies of effects of levamisole on chemotactic responses, lymphocyte function and resistance to infection have been extended and a controlled clinical trial has been designed.

Two evaluations of chemotherapeutic agents for chronic candidiasis have been conducted.

A major achievement in the laboratory has been the development of a murine model for transfer factor. The system has been adapted for transfer of delayed-type hypersensitivity to soluble proteins and in vitro correlates of cellular immunity are being compared with the in vivo responses.

Major Findings:

The lymphokine, E-RAF, is produced by mitogen-stimulated cells and by antigen-stimulated lymphocytes if the cell donor has cellular immunity to the antigen. We now have evidence that antigen-induced E-RAF and mitogen-induced E-RAF have different molecular weights. It apparently functions by enhancing maturation of "null" cells into T-cells.

E-RAF production has been studied in a patient with SCID both before and after thymus transplantation. Mitogen and antigen stimulated cells did not yield E-RAF before the transplant. By the third post-transplant day, mitogen-stimulated lymphocytes from the patient produced E-RAF. On the same day, the patients cells did not respond to mitogens with LIF production or lymphocyte transformation and he did not have normal numbers of circulating T-cells.

Most candidiasis patients have normal numbers of circulating E-rosette-forming T-cells. However, the majority of patients have somewhat low numbers of T_H cells (containing the helper cells) and excessive numbers of T_S cells (containing the suppressor cells). When the ratios of T_H/T_S are calculated 6 of 16 patients had low values, suggesting an immunoregulatory defect in these patients.

Human lymphocytes may develop suppressor activity when they are activated with concanavalin-A. In contrast, human cord blood-lymphocytes are suppressive of responses by adult cells even without concanavalin activation. Fetal cells appear to be in a "suppressive" state under normal conditions.

The work on ontogeny of lymphocyte function and resistance to oncogenesis with Ad 2-transformed cells and cell lines has been extended. All newborn hamsters are susceptable to these tumors until about 9 days of age. After this time, all animals are resistant. At about this age, spleen cells become responsive to concanavalin A; similar responses by blood lymphocytes do not appear until the fourth week of life. Of particular interest was the finding that spleen cells do not become responsive to alloantigens (in MLR) until 7-10 days after development of resistance to tumor induction. Other experiments have shown that the developmental delay is probably due to immature macrophages rather than lymphocytes.

A controlled clinical trial showed that clotrimazole buccal troches were efficacious in treating chronic oral candidiasis. A study of the systemic antifungal, ketoconazole, has been started.

The murine transfer factor system has been adapted to transfer of cell-mediated responses to ovalbumin and ferritin. The in vivo responses are compared to MIF production by antigen-stimulated cells. Studies with genetically-controlled antigens are underway.

Significance

1) These experiments and those presented in sub-project B (below) further define the relationship between cellular immune responses, inflammation and susceptibility to certain chronic infectious diseases. The relative contribution of individual lymphokines to cell mediated immune responses is unclear. For example, it is not known which or how many lymphokines are required to produce a "positive" skin test. It is also important to define the relationship of specific lymphokine production and "immunity" to infection with a microorganism. E-RAF appears to induce maturation of "null" cells into E-rosette-forming T-cells. Thus, the "null" cells are probably pre-T cells.

Proposed Course: These studies will be continued.

Objectives (sub-project B):

1) Evaluation of immunologic reconstitution as a therapeutic adjunct for treating chronic infectious diseases;

2) Identification and characterization of the components of dialyzable transfer factor and assessment of their functions in immunologic and inflammatory reactions;

3) Investigation of transfer factor-like materials in laboratory animals.

Methods Employed:

The trial of transfer factor in candidiasis is being continued with the oldest participants being in the seventh year of treatment. These subjects receive transfer factor every four months. A companion study in which other immunodeficient candidiasis patients are being treated with transfer factor from either candida-sensitive or candida-insensitive donors is underway.

Major Findings:

Thus far seven patients in whom remissions were induced with amphotericin are receiving transfer factor from candida-sensitive donors. In each case the patient has developed in vivo and in vitro evidence of cellular immunity to candida. This indicates that the transfer factor was active and that transfer factor was capable of "correcting" the immunologic lesion in the patients' cells. Four of the patients have maintained positive skin responses to candida and all of these patients have remained in remission for 3 to 8 years. These remissions are substantially longer than those expected with amphotericin alone. The three patients who failed to maintain cellular immunity to candida also suffered relapses of cutaneous candidiasis.

Five patients have received transfer factor from donors who do not have cellular immunity to candida. All patients were treated with amphotericin to induce remissions. None of the recipients developed cellular immunity to candida, an observation that suggests that transfer factor has immunologic specificity. Two of these patients have relapsed. The other three are in remission and have been for periods of 8 months to 3 years.

In summary, all recipients of transfer factor (from either candida-sensitive or candida-insensitive donors) have remissions that are longer than those seen with amphotericin B alone. It is important to note that the relapses occurred only in persons who did not achieve or maintain cell-mediated immunity to candida.

The differences between the numbers of remissions in the two groups is significant at the level of p=0.055.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00047-10 LCI

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Clinical Studies on Patients With Known or Suspected Parasitic Diseases.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PIs:	F.A.Neva	Head, Clinical Parasitology Section, LCI, NIAID (dual position)
	T.E. Nash	Senior Investigator, Clinical Parasitology Section, (dual position), LCI, NIAID
	E.A. Ottesen	Senior Investigator, Clinical Parasitology Section, (dual position), LCI, NIAID
	D.J. Wyler	Senior Investigator, Clinical Parasitology Section, (dual position), LCI, NIAID
Others:	R. D'A. Gusmão	Visiting Fellow, LPD,NIAID
	T. Lawley	Medical Investigator, NCI
	M.N. Lunde	Research Zoologist, LPD,NIAID
	J.D. Berman	Clinical Associate, LCI,NIAID
	D.M. Dwyer	Research Microbiologist, LPD,NIAID

COOPERATING UNITS (if any) Federal University of Goias, Goiania, Brazil (Profs. J. Rezende and A. Rassi); Duke University, Division of Immunology (Dr. F. Ward); Federal University of Parana, Brazil (Dr. E. Ferreira); Tuberculosis Chemotherapy Center, Madras, India (Dr. S.P. Tripathy); Medical College of Madras, India

LAB/BRANCH
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SECTION
Clinical Parasitology Section

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5 3/12	5 3/12	0

CHECK APPROPRIATE BOX(ES)

XX (a) HUMAN SUBJECTS XX (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The interesting association of absence of HLA tissue type-A1 with chronic Chagas' disease was not confirmed by an expanded study of cases, but DR (B cell) typing will still be done on frozen cells. The diversity of HLA haplotypes encountered in this Brazilian patient population corroborates what anthropologists have said of this population - namely, extreme genetic mixing.

Further study of different antigenic fractions of schistosome worms has disclosed one fraction to which antibody levels in patients correlate quite well with numbers of eggs being excreted, or intensity of infection.

Evidence for serum inhibitory factors and for suppressor cells has been found in filariasis patients characterized by unresponsiveness to filarial antigens. The role of immune complexes in filariasis patients is also being studied. Serum and cell factors were also found that contributed to antigen unresponsiveness in patients with chronic schistosomiasis.

PHS-6040
(Rev. 10-76)

Cooperating Units, cont'd:

(Prof. K.V. Thiruvengadam); University of Kentucky Medical Center, Lexington, Ky. (Dr. J. Burke).

Summary of work, cont'd:

Evidence for multiplication of several species of leishmania within phagolysosomes was found using a system of macrophages derived from human monocytes.

Project Description:

This report covers the clinical investigations carried out by the four investigators in the Clinical Parasitology Section (Drs. Neva, Nash, Ottesen and Wyler). Their experimental or non-clinical research is reported in the Annual Report from the Laboratory of Parasitic Diseases. Therefore, there will be some overlap in reporting and in publications listed. The Clinical Parasitology Section provides consultative service in clinical parasitic diseases to the Clinical Center and several members of the Section also participate with LCI Staff for clinical infectious diseases at the Clinical Center and at the Naval Medical Center. As in the past year, some of the studies were carried out in collaboration with investigators in India and in Brazil.

Pathogenesis and Immunology of leishmaniasis. While there have been many in vitro studies of parasite-macrophage interaction in leishmaniasis, human macrophages have not been used for such work. A system which would use human macrophages would obviously be a great advantage for research related to human disease with this parasite. Therefore, it was of particular interest that a system involving macrophages derived from human monocytes could be developed in which leishmanial growth could be observed and demonstrated. The parasites were shown to be present and dividing within phagolysosomes, as happens in vivo. Intracellular multiplication in such cells was found with L. donovani and with L. tropica, the parasites associated with visceral and cutaneous leishmaniasis, respectively. Such an in vitro system shows promise for studying effects of drugs as well as immunological events associated with leishmanial infections. (Wyler, Berman and Dwyer).

Possible influence of immune response on Chagas' disease. Collaborative studies with Brazilian colleagues were initiated recently on Chagas' disease as reported previously. In a preliminary investigation which involved mainly the study of blastogenic response to T. cruzi antigens in patients with various forms of chronic Chagas' disease, tissue typing at the A, B, and C loci was also carried out. This preliminary study suggested that both positive and negative correlations with certain HLA specificities occurred in patients with the chronic disease. The most striking association was that none of 18 megaesophagus patients were A-1, while this type occurred in 23 percent of 44 seronegative controls and in 28 percent of individuals with infection but no disease, all from the same area of Brazil. Therefore,

a larger study was carried out in the past year to include more cases and family members, and to freeze lymphocytes for B-cell and possible Dw typing as well. We have now studied larger groups of 40 to 50 in various clinical categories and the previous suspected HLA association is no longer present in typing at A, B and C loci. Therefore, we can only conclude that our sampling groups were not large enough for statistically significant results. The B-cell typing has not yet been done at the time this report was prepared. The geneticists and our collaborators interested in HLA are fascinated by the genetic diversity of the study population and believe they have found some new HLA specificities. But we are only sadder and wiser with the intricacies of relating HLA tissue types to parasitic diseases. (Neva, Gusmão, Ward and Brazilian collaborators).

Clinical significance of schistosome antigens. While antigenic complexity of parasites makes immunologic studies more difficult, it also provides more opportunities to dissect and examine the immunologic significance of various antigenic components. Last year we reported that the immune response in patients to the polysaccharide gut antigen showed a characteristic time course, being highest in recent infections and falling rapidly with time. This antibody response, however, did not reflect or correlate with intensity of infection. Recent work which has focused upon individual fractions of the TCA-soluble material from whole worms has disclosed a relationship between response to one of these antigenic components and numbers of eggs excreted. This antigen fraction, a glycoprotein, is but one of several fractions that make up a crude antigen reported by others as correlating with intensity of infection. But the antibody response to this specific glycoprotein fraction did not change with time after infection. Thus, antibody response to various but specific antigens of parasites, such as schistosomes, may assist in clinical evaluation of patients with the infection. With the antigens just cited, antibody levels in a patient can indicate whether the infection is recent or more than a year in duration and some indication of how heavy the infection is. (Nash and Lunde).

Immune response to helminth infections. Recently completed studies of histamine release from cells of patients with tropical pulmonary eosinophilia, a form of filarial infection without circulating microfilariae, highlighted the specific nature of the IgE-mediated allergic reaction provoked by microfilarial antigens upon contact with sensitized cells. But an equally if not more intriguing question is raised by this observation - namely, why is not histamine release going on all the time in vivo in those patients whose filariasis is associated with circulating microfilariae? There are indications that serum inhibitory factors are involved in this allergic hyporesponsiveness. Many of the patients with chronic filariasis and circulating microfilariae also exhibit both serum and adherent subpopulations of mononuclear blood cells that contribute to cell-mediated unresponsiveness to filarial antigens. (Ottesen).

The role of circulating immune complexes is also under study in patients with different clinical manifestations of filariasis. The C1q binding immune complexes that were demonstrated in patients with acute schistosomiasis

are being studied further to better characterize antigenic make-up of these complexes. (Ottesen and Lawley).

Tests for specific antibody in strongyloidiasis. A long experience with persistent strongyloides infection in an immuno-deficient patient made it possible to obtain larval antigens from this parasite. Preliminary results suggested that such antigens might be useful for serologic tests in patients. Therefore, S. stercoralis as well as other nematode larval antigens are being investigated for possible usefulness in the diagnosis of human strongyloides infection. (Neva and Burke).

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PERIOD COVERED
September 30, 1978 to October 1, 1979

TITLE OF PROJECT (80 characters or less)
The Pathophysiology of Autoimmune Hemolytic Anemia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Frank	Head, Clinical Immunology Section and Chief, LCI	LCI	NIAID
OTHER:	M. Hamburger	Clinical Associate	LCI	NIAID

COOPERATING UNITS (if any) T. Lawley (Dermatology Branch, NCI/NIH); P. Plotz (Arthritis Branch, NIAMDD/NIH); H. Moutsopoulos, (NIDR); T. Chused, (NIAID); E. Franklin, (NYU School of Medicine); G. Sharp, (Univ. of Missouri School of Medicine).

LAB/BRANCH
Laboratory of Clinical Investigation

SECTION
Clinical Immunology Section

INSTITUTE AND LOCATION
NIAID, NIH Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS x ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have continued to develop and extend the usefulness of our new technique for measuring IgG Fc receptors and complement receptors in various groups of patients with autoimmune disease. These techniques have proven useful both in diagnostic tests and for developing a further understanding of pathophysiologic processes. In the past year we have extended our studies to patients with mixed IgM, IgG cryoglobulins and have shown that these patients can be divided into two groups. The patients with Fc receptor defects are the ones who develop immune complex mediated renal disease. The patients with normal Fc receptor function do not appear to develop immune complex mediated renal disease. The follow-up studies have been performed in our patients with lupus erythematosus and it has been shown that as the patients improve the immune clearance defect also improves. Patients with mixed connective tissue disease have been studied and have been shown in general to be free of defects in immune clearance not withstanding the fact that they have circulating immune complexes. This is in keeping with previous observations that these patients do not develop immune complex mediated tissue injury.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To define the pathophysiologic function of antibody and complement on the surface of red cells in patients with hemolytic anemia, and to use the findings which have been developed from this study to develop new tests for immunologically specific receptors function in patients with a variety of disease states. The tests developed for the study of autoimmune hemolytic anemia have been found to have wide application to other autoimmune and immunologic problems.

Methods Employed:

These studies examine the clearance of radiochromated erythrocytes from the circulation. The initial studies were performed in a guinea pig model, but all of the recent studies have been performed in human volunteers or in patients with various diseases. In the initial studies in humans, the clearance of erythrocytes coated with IgM isoagglutinin antibodies were examined. In later studies radiochromated erythrocytes were coated with highly purified human IgG anti-Rh antibodies and the survival of these cells was also studied. The third general set of clearance studies examined the clearance of radioactive albumin which had been aggregated. This is the standard method for determining RES clearance and can be used to determine both liver blood flow and the ability of the reticuloendothelial system to clear particulate materials.

Major Findings:

In past years, we identified the immunologically active protein fragments which, when deposited on red cells, are responsible for the clearance of those cells from the circulation. It was reported that receptors for these immunologically specific fragments were present on the membrane of macrophages which were responsible for removal of cells coated with these fragments from the circulation. It became clear that it was possible to study patients with a wide variety of diseases to determine whether their receptors for these immunologically active fragments were functioning normally in vivo. It has been known for years that the reticuloendothelial system (RES) plays a major role in removing foreign material from the circulation. These foreign materials include bacteria and parasites, as well as endogenously generated immunoreactive materials such as immune complexes. It was suspected that defects in RES function might be important in both infectious and immunologic diseases. However, when this question was examined directly using the techniques available, no defects in RES function were noted in any disease state in which blood flow to the RES organs was normal. Until the present studies, the technique available for examination of RES function was that of study of clearance of aggregated albumin from the circulation. This study has led to the development of new techniques for evaluation of RES function. Using these new techniques developed within our unit we have shown that there are indeed diseases with defects in RES function if one examines this question looking at specific re-

ceptors for immunologically active material. Thus receptors for complement and immunoglobulin on a patient's macrophages will recognize these immunologically active materials coating foreign substances as they circulate.

The defects are quite specific for different sets of receptors. Our test utilizes the patient's own red blood cells. The cells are coated with antibody and/or complement fragments and are infused into the patient and their clearance is followed. Using this technique we have found that almost every patient with active lupus erythematosus has a major defect in Fc receptor specific clearance although the clearance of aggregated materials is normal. We believe that it is quite likely that this clearance defect extends to the clearance of the circulating immune complexes which occur in these patients. If such complexes are not cleared efficiently from the circulation, they can be deposited in glomeruli where they can cause immune damage. We find that as the disease improves, the clearance defect also tends to improve and the comlexes present in the circulation tend to disappear. However it is striking that most patients with lupus erythematosus have a persistence of their clearance defect for long periods of time, even on treatment. Our studies in lupus erythematosus have already been confirmed by a group in London. These initial findings in lupus were published in the past year in the New England Journal of Medicine.

Extensive studies have been conducted of Sjögren's Syndrome. These studies demonstrated that patients with clearance defects in the presence of circulating immune complexes tend to develop peripheral manifestations of their Sjögren's Syndrome including autoimmune disease and extensive tissue injury. On the other hand patients who do not have clearance defects tend not to develop secondary manifestations of their Sjögren's Syndrome with peripheral tissue destruction even in the presence of circulating immune complexes. This material is in press in the Annals of Internal Medicine. We have now had an opportunity to analyze the circulating immune complexes in patients with Sjögren's Syndrome and we have shown that a very high proportion of patients have immune complexes and that these complexes are of multiple types. Moreover, the rheumatoid factor present in these patients is only one type of complex and is not responsible for many of the positive tests for immune complexes shown in the studies of the serum of these patients.

In the past year with Dr. Edward Franklin and his group at NYU we have had an opportunity to study patients with IgM-IgG mixed cryoglobulinemia. These patients have been shown to fall into two groups with regard to the development of peripheral manifestation of disease in spite of the fact that all have circulating immune complexes. One group of patients has been shown to possess normal Fc receptor function and these patients do not develop certain severe peripheral manifestations of disease such as immune complex mediated glomerulonephritis. A second group of patients have been shown to have a clearance defect and it is these patients that have evidence of glomerulonephritis. Once again the usefulness of the clearance studies in determining the nature of the pathophysiologic process in this disease is clear.

We have also studied a large group of patients with mixed connective tissue disease with Drs. Gordon Sharp and Harry Moutsopoulos. These patients tend not to have tissue deposition of immune complexes and extensive tissue damage although they have circulating immune complexes in quantity. Interestingly, our studies show little or no evidence of a clearance defect in these patients except for those who have many of the manifestations of systemic lupus erythematosus. The latter patients tend to resemble patients with lupus erythematosus in that they have anti-DNA etc. and interestingly these patients do have clearance defects. Finally, we have studied a group of patients with rheumatoid arthritis. These patients do not have striking Fc receptor clearance defects suggesting that much of the immunopathology that occurs is not systemic immune complex mediated injury but originates in the joints themselves.

Significance to Biomedical Research

These techniques allow for the complete reevaluation of reticuloendothelial system function in man. Already they are being widely applied. The use of these techniques has already allowed for the description of the first patients with receptor specific defects. C3b specific defects have been demonstrated in primary biliary cirrhosis and Fc receptor defects in a number of serious autoimmune diseases. These techniques provide a powerful new tool in the evaluation of these patient groups and provide a new approach to understanding the pathophysiologic basis of these diseases.

Proposed Course:

We intend to extend these studies to additional autoimmune diseases. Moreover, we intend to attempt to determine whether patients with various kinds of infectious disease may also have clearance defects which contribute to their inability to clear bacteria from the circulation. At the present time we are examining the possibility of studying patients with sickle cell anemia to determine whether these patients have a clearance defect that might help account for their propensity to develop overwhelming sepsis.

Publications:

1. Jones, E.A., Frank, M.M., Jaffe, C.J., and Vierling, J.M.: Primary Biliary Cirrhosis and the Complement System. Ann. Int. Med.: In press.

2. Jaffe, C.J., Vierling, J.M., Jones, E.A., Lawley, T. and Frank, M.M.: Receptor specific clearance by the reticuloendothelial system in chronic liver diseases: Demonstration of defective C3b specific clearance in primary biliary cirrhosis. J. Clin. Invest. November 1978: 62:1069-1077, 1978.

3. Lawley, T., Moutsopoulos, H.M., Katz, S.I., Theofilopoulos, A.N., Chused, T.M., and Frank, M.M.: Circulating Immune Complexes in Sjogren's Syndrome. J. of Immun. In press.

4. Frank, M.M., Hamburger, M.I., Lawley, T.J., Kimberly, R.P., and Plotz, P.H.: Defective Reticuloendothelial System Fc Receptor Function in Systemic Lupus Erythematosus. N. Engl. J. Med. 300: 518-523, March, 1979.

5. Moutsopoulos, H.M., Chused, T.M., Mann, D.L., Klippel, J.H., Fauci, A.S., Frank, M.M., Lawley, T.J. and Hamburger, M.I.: Sjogren's Syndrome (Sicca Syndrome): Current Issues. An edited transcript of a Combined Clinical Staff Conference of the Clinical Center, Bethesda, Maryland, April 19, 1979.

6. Hamburger, M.I., Moutsopoulos, H.M., Lawley, T. and Frank, M.M.: A defect in Reticuloendothelial System Fc Receptor Function in Sjogren's Syndrome. Ann. Int. Med. In press.

7. Hamburger, M.I., Gorevic, P., Franklin, E.C. and Frank, M.M.: The Function of the Reticuloendothelial System in Autoimmune Disease. Transaction of the Association of the American Physicians. 1979. In press.

8. Frank, M.M.: The Function of the Reticuloendothelial System in Autoimmune Diseases. In. Immune Mechanisms in Renal Disease. Cummings, N. (Ed.) In press.

9. Frank, M.M.: Reticuloendothelial Complement-Specific Clearance of Circulating Particles. p. 77-8 In Jones, E.A. (Moderator). Primary Biliary Cirrhosis and the Complement System. Ann. Intern. Med. 90: 72-84, 1979.

10. Frank, M.M.: The Role of Complement in the in vivo Destruction of Erythrocytes. In. Proceedings of the American Red Cross Eleventh Annual Scientific Symposium. In press.

11. Frank, M.M.: The Role of the Reticuloendothelial system in the Clearance of Circulating Cells Coated with Antibody and Complement. In: Proceedings of the WHO Meeting on the Role of the Spleen in the Immunology of Parasitic Diseases. In press.

12. Frank, M.M., Atkinson, J. and Jaffe, C.: The Role of Antibody and Complement in Immune Clearance of Erythrocytes in Man. In Clinical Aspects of the Complement System: International Symposium. Opferkuch, W., Rother, K. and Schultz, D.R. (Eds) Georg Thieme Publ., 1978, pp. 70-76.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00049-09 LCI

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Initiation and Regulation of Antigen Recognition

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M.M. Frank	Acting Head, Biologic Structure Section	LCI NIAID
OTHER:	J.T. Blake	Bio. Lab. Tech. (Micro.)	LCI NIAID
	K.U. Cehrs	Bio. Lab. Tech. (Micro.)	LCI NIAID
	K. Yokomuro	Guest Worker	LCI NIAID
	J. Schroer	Guest Worker	LCI NIAID
	J. Thomas	Clinical Associate	LCI NIAID
	R. Clark	Clinical Associate	LCI NIAID

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Clinical Investigation

SECTION
Biologic Structure Section

INSTITUTE AND LOCATION
NIAID/NIH Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5 11/12	4 5/12	1 6/12

CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The interest of the Biologic Structure Section is to characterize the role of the macrophage in antigen recognition by T lymphocytes in the expression and initiation of cell-mediated immunity in man and experimental animals. The Section has been interested in exploring the role of the major histocompatibility complex in the genetic regulation of cell-cell interactions required for expression of cell mediated and humoral immunity. The Biologic Structure Section is also actively characterizing the physical state, localization, and fate of macrophage associated antigen. Additional studies of genetics of immune responsiveness to insulin done in man are assessed and compared to mouse and guinea pig. Determinant selection by macrophages functionally describes Ir gene control of antigen recognition by T lymphocytes in experimental animals. An understanding of the operation of a similar mechanism in man may have implications in the pathophysiology of insulin allergy in diabetics.

Project Description:

Objectives:

1) To characterize in a quantitative and qualitative fashion the role of macrophages and lymphocytes in the development of cellular immunity.

2) To study in experimental animals the mechanism of lymphocyte antigen recognition using defined antigens such as insulin.

3) To study the cellular and molecular basis of the genetic control of immune responsiveness and the role of surface membrane determinants in cell cooperation.

Methods Employed:

Standard assays have been developed for:

1) Determination of DNA synthesis of mouse and guinea pig lymphocyte populations.

2) Preparation and isolation of partially purified populations of lymphocytes.

3) Column purification of cells and proteins.

4) Binding of lymphocytes to macrophage receptors.

5) The uptake and fate in culture of soluble protein antigens.

Major Findings:

Dr. Alan Rosenthal left the NIH last year to assume a position as Director of Immunology of Merck, Sharp and Dohme. This year's efforts have been directed at completing projects of his fellows who were committed to an additional years tenure.

Members of this Laboratory have begun to characterize seven anti-insulin antibody secreting cell lines prepared by cell fusion techniques in collaboration with Dr. K. Jin Kin. Several interesting results were obtained from these studies. An autoreactive antibody which binds to a mouse's own insulin was one of the seven isolated. Furthermore, the precise amino acid sequence of the binding site on the insulin molecule (region of A chain residues 8-10) of another anti-insulin fusion product was identified. This last antibody can distinguish beef insulin from pork insulin, rat insulin or other insulins which differ in amino acid sequence in this region of the insulin molecule. In collaboration with Dr. James W. Thomas, the kinetics, specificity and Ir gene control of the plaque forming cell response to insulin and TNP-insulin in the mouse were also completed and submitted for publication.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00050-09 LCI

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Clinical Studies of Complement Abnormalities of Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Frank	Head, Clinical Immunology Section and Chief LCI	LCI	NIAID
OTHER:	S. Hosea	Clinical Associate	LCI	NIAID
	K. Katusha	Microbiologist	LCI	NIAID
	M. Santaella	Guest Researcher on IPA	LCI	NIAID

COOPERATING UNITS (if any)
D.W. Alling, OSD, NIAID, NIH): D. Triantaphylapolis and M. Wickerhauser
American Red Cross, Bethesda, MD

LAB/BRANCH
Laboratory of Clinical Investigation

SECTION
Clinical Immunology

INSTITUTE AND LOCATION
NIH/NIAID, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3 8/12	3 2/12	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Hereditary Angioedema is an autosomal dominant disease characterized by abnormalities in C1 esterase inhibitor. We have previously shown that danazol corrected the protein abnormality in patients with this disease. During this year we have further extended observations of the usefulness of danazol and danazol toxicity in a large patient population. We have begun to characterize the incidence of autoimmune diseases in this patient group as well. Further, we have extended our experience with the use of purified C1 esterase inhibitor in the treatment of Hereditary Angioedema attacks and have shown that the therapy can abort these potentially life threatening attacks.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

The general objective of this program has been to define the role of complement in human illness and to develop better methods of therapy for immune damage mediated by complement. As in the past year, major emphasis was placed on our studies with patients with hereditary angioedema. This illness, which carries a high mortality rate, is particularly useful for study since it can serve as a model for complement-mediated immune damage in man and as model for the study of genetically controlled autosomal dominant diseases. We were particularly interested this year in defining the usefulness and toxicity of danazol in a large patient population. We were also interested in extending our efforts to stop attacks of hereditary angioedema after they have already begun. Danazol does not work in this situation.

Methods Employed:

Purified complement components are prepared, and antisera to various complement components and component inhibitors are used for estimation of component levels in human sera and other body fluids. Double-blind therapeutic studies are employed to test new methods of therapy. Patients on experimental drug protocols are followed to determine signs of toxicity.

Major Findings:

In the past years, we have developed and evaluated a new drug danazol for treatment of hereditary angioedema. Earlier we showed that this drug is markedly effective in treating this disease and is relatively non-toxic. The drug has the striking effect of causing patients with the deficient serum inhibitor protein to synthesize this protein so that levels of the protein in blood return to normal. With the restoration of normal levels of the protein in blood, the patient corrects his biochemical abnormalities and thereafter is free of symptoms. The long-term use of this new drug has never been studied in any patient population and it was essential to begin to define those situations in which the drug was useful or on the other hand was likely to cause problems. We also were interested in attempting to stop attacks of hereditary angioedema once they had begun. It takes days for danazol to have its effect. Patients who are not on danazol however, will often present to an emergency ward having severe angioedema which may be life-threatening. At the present time there is no safe method of treating such patients. The use of fresh, frozen plasma has been advocated, since fresh plasma has the protein which the patients are missing; however, fresh plasma also has complement components in it and the patients are depleted of these components at the time of an attack. It has been suggested that the infusion of complement components will make these patients much more ill before they improve. Thus, fresh plasma is not advocated for use in this disease.

Twenty-seven new patients were seen with Hereditary Angioedema in 1978 and 8 additional new patients were seen in 1979. We now have 63 patients on danazol therapy, 7 on oxymethylone, 4 on EACA, 9 on methyltestosterone. We have developed considerable further experience with danazol toxicity and are now the leading group in developing methods to evaluate danazol toxicity. We are also now the largest group following patients with HAE on therapy and have begun to pull together our studies of the incidence of autoimmune disease in this interesting disease. We now have several patients with autoimmune thyroiditis, glomerulonephritis, regional enteritis, ulcerative colitis, pancreatitis, etc. We are initiating studies to assess how the complement deficiency state predisposes to autoimmune disease.

Our infusion studies are also progressing in a highly satisfactory way, however, we are still having difficulty obtaining large quantities of the C1 esterase inhibitor from the Red Cross. We have assisted the Red Cross in preparation and in evaluation of C1 esterase inhibition. Moreover, we have now completed a series of infusions of purified C1 esterase inhibitor into patients with HAE at times when they were not experiencing attacks and also have infused the protein into patients in the midst of attacks. These studies have shown that C1 esterase inhibitor is quite benign and does not carry the risk of hepatitis. When infused into patients free of clinical symptoms these patients experience an elevation of C1 esterase inhibitor and a subsequent elevation of C4 levels as might be expected from pathophysiologic mechanism of this disease process. When infused into patients experiencing attacks of HAE, attacks, have been aborted in as short a period as 5 minutes and as long a period as several hours. It would appear that this will afford an excellent approach in emergency rooms for treating patients with life-threatening attacks of Hereditary Angioedema.

Significance to Medical Research

Our laboratory has been the focus for most of the recent therapeutic developments in current treatment of this disease. Patients with hereditary angioedema experience a 25% mortality and have extensive morbidity from their constant attacks of the disease. As a result of our therapeutic interventions these patients lead a normal life. Moreover our development of drugs and our study of their mechanism of action has led to a far greater understanding of the genetic basis of the protein abnormality underlying the disease. At present, this disease represents one of the few genetically controlled disorders in which there has been biochemical correction and thereby cure of a severe illness.

Proposed Course:

We are continuing to evaluate the long term use of drugs in patients with this particular disease. We are also gradually increasing the number of other patients with other complement-related abnormalities which are under study. These include such diverse illnesses as autoimmune hemolytic anemia and systemic lupus erythematosus.

Publications:

1. Gelfand, J.A., Rosa, G.R., Conley, C.L., Allen, J.C., Reinhart, R., Humphrey, R.L. and Frank, M.M.: Acquired Cl Esterase Inhibitor Deficiency and Angioedema. Medicine 58: 321-28, 1979.

2. Levinson, A.I., Summers, R.J., Lawley, T.J., Evans, R., III, and Frank, M.M.: Evaluation of the Adverse Effects of Long-Term Hyposensitization. J. Allergy Clin. Immunol. In press.

3. Atkinson, J.P. and Frank, M.M.: The Complement System. In Parker, C. (Ed.): Clinical Immunol. Philadelphia, PA, W.B. Saunders Co. In press.

4. Parillo, J.E., Lawley, T.J., Frank, M.M., Kaplan, A.P. and Fauci, A.S.: Immunologic Reactivity in the Hypereosinophilic Syndrome. J. Allergy Clin. Immunol. In press.

5. Frank, M.M.: The Effect of Sex Hormones on a Complement Related Clinical Disorder, Hereditary Angioedema. Proceedings of the Kroc Foundation Meeting on Sex Factors, Steroid Hormones and the Host Response. Amacher, P. and Talal, N. (Eds.). In press.

6. Gadek, J.E., Hosea, S.W., Gelfand, J.A. and Frank, M.M.: Response of Variant Hereditary Angioedema Phenotypes to Danazol Therapy: Genetic Implications. J. Clin. Invest. 64: 280-286, July, 1979.

7. Frank, M.M. and Hosea, S.W.: Complement. In Cohen, A.S. (Ed.): The Science and Practice of Clinical Medicine. New York: Grune and Stratton, Inc. 1979, p. 393.

8. Hosea, S.W. and Frank, M.M.: Differential Diagnosis of Hypocomplementemia. In Cohen, A.S. (Ed.): The Science and Practice of Clinical Medicine. New York: Grune and Stratton, Inc. 1979, p. 432.

9. Lawley, T.J. and Frank, M.M.: Immune Complexes and Immune Complex Mediated Diseases. In Parker, C. (Ed.): Clinical Immunology. In press.

10. Frank, M.M., Gelfand, J.A., Sherins, R.J., Alling, D.W., and Gadek, J.: The treatment of hereditary angioedema with danazol. In Clinical Aspects of the Complement System: International Symposium. Opferkuch, W., Rother, K. and Schultz, D.R. (Eds.) Georg Thieme Publ., 1978, pp. 134-138.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00051-07 LCI

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Host Defense Mechanism Against Pseudomonas Infection in Normal and Immunosuppressed Hosts

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.P. Aduan	Medical Officer	LCI NIAID
OTHER:	E. Harvey	Bio. Lab Tech. (Micro.)	LCI NIAID

COOPERATING UNITS (if any)
A.S. Levin, A.B. Deisserroth, and F.R. Applebaum (Pediatric Oncology Branch, NCI, NIH)

LAB/BRANCH
Laboratory of Clinical Investigation

SECTION
Bacterial Disease Section

INSTITUTE AND LOCATION
NIAID, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2 3/12	1 3/12	1

CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

INACTIVE DURING CURRENT YEAR. TERMINATED.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00054-07 LCI

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

The Etiology and Pathogenesis of Viral Gastrointestinal and Respiratory Tract Infections in Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. Dolin	Head, Medical Virology Section (until January 1, 1979)	LCI NIAID
OTHER:	R. Berg	Clinical Associate	LCI NIAID
	R. Schooley	Clinical Associate	LCI NIAID

COOPERATING UNITS (if any)
A.Z. Kapikian (LID/NIAID/NIH); R.G.Wyatt (LID/NIAID/NIH); B.R. Murphy (LID/NIAID/NIH).

LAB/BRANCH
Laboratory of Clinical Investigation

SECTION
Medical Virology Section

INSTITUTE AND LOCATION
NIAID/NIH Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6/12	6/12	-

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

TERMINATE THIS PROJECT

Studies of the etiology and pathogenesis of viral gastrointestinal tract infections in man continued until the departure of the Principal Investigator.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

1) To investigate the pathogenesis of and host response to viral infections of the respiratory and gastrointestinal tracts in man. Experimentally induced disease in normal volunteers, as well as naturally occurring cases are studied.

2) To define the biologic, immunologic, and epidemiologic properties of the etiologic agents of viral gastroenteritis in man.

Methods Employed:

Viral agents were obtained from naturally occurring outbreaks of gastroenteritis and respiratory infection. The pathogenesis and host responses to these agents were evaluated in *in vitro* systems and in volunteer studies.

Major Findings:

Characterization of the small 27-32nm gastroenteritis agents continued in volunteer studies. Investigations of the cell-mediated immune responses to influenza vaccines were completed.

Significance to Biomedical Research and the Program of the Institute:

Viral respiratory and gastrointestinal tract infections are the most common disease experiences in American families and affect all age groups of a broad segment of population. As yet, there are no adequate control measures available. Identification and characterization of etiologic agents, as well as studies of the pathogenesis of these diseases in man, should form the basis for rational development of methods of prevention and treatment.

Proposed Course:

This study has been terminated upon the departure of the Principal Investigator.

Publications:

1. Dolin, R.: Antiviral compounds in viral infection of the gastrointestinal tract. In Buchanan, R., Galasso, G. and Merigan, T. (Eds.): Antivirals in Man. In press.

2. Dolin, R.: Viral gastroenteritis. In Beeson, P.B., McDermott, N. and Wyngaarden, J.B. (Eds.) Textbook of Medicine 15th Edition, Philadelphia, Pa., W.B. Saunders. In press.

3. Dolin, R.: Norwalk-like agents of viral gastroenteritis. In Mandell, G., Douglas, R.G., Bennett, J.E. (Eds.) Principles and Practice of Infectious Diseases, New York, Wiley and Sons. In Press.

4. Reichman, R.C., Pons, V.G., Murphy, B.R., Caplan, E.A. and Dolin, R.: Cell mediated cytotoxicity following influenza infection and vaccination in humans. J. Med. Virol. In press.

5. Pons, V.G., Reinertsen, J.R., Steinberg, A.D. and Dolin, R.: Decreased cell mediated cytotoxicity against virus-infected cells in systemic lupus erythematosus. J. Med. Virol. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00055-07 LCI

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Regulation of the Immune Response in Man and Experimental Animals

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. S. Fauci	Head, Clinical Physiology Section	LCI NIAID
		Deputy Clinical Director	NIAID
Other:	C. Burch	Biologist	LCI NIAID
	T. Cupps	Clinical Associate	LCI NIAID
	A. Dimitriu	Guest Worker	LCI NIAID
	B. F. Haynes	Senior Investigator	LCI NIAID
	P. Katz	Clinical Associate	LCI NIAID
	H. C. Stevenson	Clinical Associate	LCI NIAID
	D. Volkman	Clinical Associate	LCI NIAID
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COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Clinical Investigation

SECTION
Clinical Physiology Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6 5/12	4 5/12	2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The precise mechanisms and immunoregulatory events associated with triggering of human B lymphocytes following non-specific and antigen induced stimulation have been delineated. Functionally distinct as well as overlapping immunoregulatory lymphocyte subsets have been characterized. The precise abnormalities of immunologic reactivity have been characterized in several immunologically mediated diseases such as systemic lupus erythematosus, Sjögren's syndrome, acute infectious mononucleosis, sarcoidosis and tuberculosis. For the first time, an anti-idiotypic antibody has been produced which specifically blocks the function of human B cells bearing the corresponding idiotype. Hybridoma lymphocyte cell lines have been established which are secreting antibody which binds specifically to functionally distinct subsets of human blood lymphocytes. Counter-current centrifugation techniques have been adapted which result in 95% and greater purity and yield of monocytes from human blood. These cells have been characterized functionally and structurally. Modulation of lymphocyte subsets by a number of clinically relevant pharmacologic agents have been defined. Clinical therapeutic studies have yielded extraordinarily favorable results in the cytotoxic drug approach to the systemic necrotizing vasculitides.

Project Description

Objectives:

1) To delineate the mechanisms of regulation of immunologic reactivity by purified subpopulations of human lymphoid cells. To examine the precise functional capabilities of these individual lymphoid cell subpopulations as well as the mechanisms whereby they maintain the normal homeostatis of positive and negative signals which regulate normal immunologic reactivity.

2) To determine the role of these lymphoid cell subpopulations in the pathogenesis of immunologically mediated diseases such as the vasculitides, hypersensitivity diseases, granulomatous diseases, the spectrum of autoimmune diseases, as well as infectious diseases characterized by aberrations of immunologic reactivity. In particular, to delineate the alterations of various subpopulations of cells by mechanisms such as a) modulation of cell surface receptors (for example, Fc receptors) by immune complexes, cryoglobulins or other serum factors; b) activation of cell subsets by viruses (such as Epstein-Barr) virus); and c) depletion, proliferation, or redistribution of cell subsets among lymphoid compartments. In this regard, to determine the role of these mechanisms in the abnormalities of immunoregulation characteristic of these diseases.

3) To develop hybridoma cell lines secreting monoclonal antibodies directed against stable lymphoid cell surface antigens which define functionally distinct subpopulations of immunoregulatory cells.

4) To determine the role and mechanisms of anti-idiotypic antibodies in the regulation of human B cell responses in normal immune reactivity and in diseases characterized by the production of abnormal serum components (M components).

5) To investigate the cellular interactions, molecular, and biochemical events associated with triggering of various mononuclear cell subpopulations and to determine the relationship between selective triggering via various cell surface receptors and the kinetics of expression of various functional capabilities of the given cell populations.

6) To continue to develop and further perfect culture and assay systems for primary in vitro activation of human B lymphocytes to antibody production following polyclonal activation or specific antigenic stimulation.

7) To develop an in vitro model of autoreactivity by selective triggering or depletion of immunoregulatory cell subpopulations in order to define more precisely the primary or secondary role of altered immunoregulation in autoimmune diseases.

8) To delineate the mechanisms whereby recognition of self molecules (such as Ia-like determinants) plays a role in non-self antigenic recognition. In this regard, to determine the role of autoreactivity in the normal immunologic homeostasis in man and to characterize the aberrancies of this otherwise normal level of autoreactivity which are associated with pathologic autoimmunity and altered immune surveillance in certain neoplastic diseases, particularly those of the lymphoproliferative type.

9) To develop methodologies of fractionating purified populations of human peripheral blood monocytes and T cell subsets by elutriation centrifugation techniques in order to avoid pre-activation of the respective populations which invariably occurs with positive selection methodologies.

10) To induce, purify and characterize soluble factors from purified subpopulations of human lymphocytes and monocytes which are involved in the regulation of immunologic reactivity.

11) To delineate the precise mechanisms whereby immunosuppressive agents such as corticosteroids and cytotoxic drugs affect the immune responses in man specifically examining their selective effects on subpopulations of lymphocytes and monocytes with regard to their circulatory kinetics, cellular interactions, activation, and expression of in vivo and in vitro functional capabilities. In addition, to correlate the selective effects of corticosteroids and cytotoxic drugs on these various parameters with the suppression of disease activity in various inflammatory and immunogically-mediated disease states in man, and by doing to aim at a greater understanding and therapeutic specificity in the clinical use of these agents. Furthermore, to study the mechanisms whereby manipulations of immune reactivity as described above affect host defenses against infection, tumor surveillance and propensity towards autoimmune states.

12) To study the functional capabilities of eosinophils and mononuclear cells in the idiopathic hypereosinophilic syndrome in order to elucidate the mechanisms whereby these cells cause invasion of tissue and subsequent tissue damage in this disease. To use this information to gain a greater understanding of the functional properties of eosinophils in normal states, various diseases and to determine their sensitivity to various therapeutic regimens.

13) To extend the concept of immunoregulation to the effect of the immune system on other cell types. Specifically, to study the mechanisms of immunoregulation by lymphoid cell subpopulations on eosinophil and neutrophil kinetics and function in the hypereosinophilic syndrome and certain neutropenic states respectively.

14) To continue to study the spectrum of the vasculitides in man from a mechanistic, pathophysiologic, clinical and therapeutic standpoint.

Methods Employed:

1) The predominant theme of this laboratory is the delineation of the

mechanisms of immunoregulation in normal human immune reactivity and in diseases characterized by abnormalities of immunoregulation.

In this regard, a major component of the methodology relates to the fractionation, identification and purification of immunoregulatory lymphoid cell subpopulations and the in vitro culturing of these cells to determine their diverse functional capabilities. Subpopulations of cells are identified predominantly by surface markers and functional capabilities are assessed by a variety of in vitro assays. We have focused on the relationship between various in vivo and in vitro triggering signals and the subsequent in vivo expression of direct and immunoregulatory functional capabilities. This has been focused predominantly in the area of activation, proliferation and differentiation of human B lymphocytes to antibody producing and secreting cells. In this regard, we have been utilizing a unique model of human B cell function with was originally developed in this laboratory. It is a system of primary in vitro stimulation of bone marrow derived (B) lymphocytes by polyclonal activation as well as specific antigenic stimulation with subsequent measurement of single cell antibody production by a direct hemolysis-in-gel plaque forming cell (PFC) assay. We have further extended this system to now measure indirect (IgG) PFC. In addition, we also have developed a system for the measurement of Ig secreting cells of all classes by use of staphylococcal protein A (SPA) coated target erythrocytes together with class specific developing antisera.

Using these models, we have developed a system for the selective generation of suppressor cells or helper cells capable of modulating in vitro antibody production. In addition, we have produced soluble mediators of B cell function in man and have characterized the cell types of their origin.

Other assays employed to delineate the functional capabilities of lymphoid cells are the in vitro blastogenic responses to mitogenic and antigenic stimulation, elaboration of various soluble mediators, cell mediated cytotoxicity against various autologous, allogeneic and xenogeneic targets. Various cytotoxicity assays include spontaneous, mitogen dependent, and antibody dependent cellular cytotoxicity. A particular aim is to identify and characterize the abnormalities of lymphocyte or monocyte subpopulations involved in immunologically mediated diseases to determine the primary or secondary nature of these abnormalities and their relationship to the altered state of immune reactivity, as well as to create in vitro models of altered immunoregulation by selectively triggering or manipulating immunoregulatory subpopulations of cells.

2) Highly sensitive assays (enzyme linked immunosorbent - ELISA) have been developed and are being employed to measure the in vitro production of specific antibody following in vivo immunization of human subjects as well as primary in vitro antigenic stimulation.

3) Antisera against purified subpopulations of human lymphoid cells have been developed in order to identify, isolate, characterize, inhibit functional capabilities or deplete these functionally distinct subsets from various cell suspensions.

4) In relationship to #3, hybridoma cell lines have been developed which are producing large quantities of monoclonal antibodies directed against functionally distinct lymphocyte subsets.

5) Recently developed elutriation centrifugation techniques are being employed to isolate purified (greater than 95%) populations of human monocytes from peripheral blood, thus avoiding adherence techniques.

6) Methods to fractionate purified populations of eosinophils (90-98% pure) from human blood have been developed and are being employed.

7) Corticosteroids are administered to normal volunteers and patients requiring this drug and selective effects of these agents on the compartmentalization and functional capabilities of monocytes and lymphocyte subpopulations are determined. In addition, the differential effects of in vitro corticosteroids and in vitro irradiation on various lymphoid cell functional capabilities such as triggering, proliferation, differentiation, antibody production and secretion are being studied.

8) A subject's lymphoid cells are labeled with ^{51}chromium and reinfused back into their circulation to determine the effects of disease states and of chemotherapeutic agents on the viability and circulation patterns of these cells.

9) Bone marrow aspirates are performed in normal subjects and patients with various diseases prior to, during and following therapy. Purified populations of lymphoid cells are obtained from these bone marrow cell suspensions by various separation techniques in order to delineate the immunocompetence of bone marrow lymphoid cells in normal man, as well as the alteration of compartmentalization and function of these cells in untreated and treated disease states.

Major Findings and Significance to Biomedical Research and the Program of the Institute:

A number of significant findings in human immunobiologic research have emerged from this laboratory over the past year. These have been in several general areas including mechanisms of triggering of human B lymphocytes; immunoregulation of B and T cell function; identification and purification of functionally distinct immunoregulatory and effector lymphocyte and monocyte subpopulations in normal individuals and a characterization of the aberrations of such subsets in certain disease states; and finally studies involving the

pathogenesis and therapeutic approach to diseases characterized by abnormalities of immunologic reactivity. Specific findings of particular relevance include:

1) Major advances have been made in the delineation of the mechanisms of activation of human B lymphocytes. Mitogen induced and antigen specific in vitro assays of B cell function have been originally developed and are now being extended in this laboratory. These include the first polyclonally induced and antigen induced direct and indirect hemolytic plaque forming cell (PFC) assay for human B cells. In addition, a highly sensitive enzyme linked immunosorbent (ELISA) assay has been developed to detect minute quantities of supernatant Ig and specific antibody from in vitro cultures of human lymphocytes. These innovative assay systems are being used to delineate the complex mechanisms of immunoregulation of human B cell function in normal individuals and in disease states, as will be described below. Representatives from numerous laboratories throughout the world have visited our laboratory this year to learn these unique methodologies, and have been successful in instituting these systems in their own respective laboratories. Of particular note in this regard is the fact that in 1978 an international workshop on human B cell function was organized and directed by the Head, Clinical Physiology Section, LCI, NIAID. The purpose was to bring together the world's leading investigators in this area and compile a state of the art publication with guidelines for furture research. This led to the extremely well received book "Antibody Production in Man. In Vitro Synthesis and Clinical Implications" Edited by A. S. Fauci and R. E. Ballieux, Academic Press, New York, 1979.

2) Precise delineation of immunoregulatory lymphocyte subpopulations in normal individuals has been accomplished. We have previously reported the original description of a delicate balance between helper and suppressor cell subpopulations in the modulation of normal B cell immunologic reactivity. In addition, we have demonstrated that certain otherwise normal individuals are hypo-responders in these in vitro B cell systems, and that this hyporesponsiveness resulted directly from overly active or triggered suppressor cells. We have now extended these studies to precisely characterize the identity and nature of this naturally occuring suppressor T cell.

In addition, we have characterized the precise identity, kinetics and mechanisms of action of the mitogen (Con-A) induced suppressor cell in normal humans.

3) Applying the abovementioned systems of immunoregulation we have characterized the primary and secondary immunoregulatory abnormalities in a number of immunologically mediated diseases.

We have demonstrated the presence of altered proportions and absolute numbers of immunoregulatory T cell subpopulations in patients with systemic lupus erythematosus (SLE). In addition, we have demonstrated a deficiency of suppressor cell function in SLE patients. In this regard, an in vitro model of pre-triggering suppressor cells by activation with immune complexes has been developed to support the hypothesis of immune complex associated alterations

of immunoregulation in SLE and related syndromes. We have defined the spectrum of relative alterations of immunologic reactivity by demonstrating the presence of mild hyperreactivity of B cells associated with reversible alterations of immunoregulatory T cell subsets in Sjögren's syndrome (SS) without other associated connective tissue disorders; modest to severe B cell hyperreactivity with irreversible alterations of immunoregulatory T cell subsets in SS associated with mild SLE; and severe B cell hyperreactivity together with alterations and depletions of immunoreguatory T cell subsets in frank SLE.

Of major inportance is the fact that we have developed an anti-idiotypic antibody against the surface IgM and serum monoclonal IgM peak of a patient with chronic lymphocytic leukemia whose leukemic B cell clone is secreting monoclonal antibody against SRBC determinants. The anti-idiotypic antibody reacts with the patient's B cell surface IgM and serum IgM but not with normal B cell surface IgM or serum IgM. Furthermore, the anti-idiotypic antibody blocks the spontaneous and mitogen induced secretion of anti-SRBC antibody by the patient's B cells but not by normal B cells. This is the first demonstration in man of the inhibition of B cell function by anti-idiotypic antibody and has obvious important implications in the understanding of the role of the idiotype network in the regulation of human immunologic reactivity.

We have demonstrated the presence of spontaneously activated B cells in Epstein-Barr virus (EBV)-induced acute infectious mononucleosis (IM). We have further shown that this is due to the direct triggering of B cells by EBV via surface receptors. We have characterized the atypical lymphocyte in IM and have demonstrated for the first time the emergence of suppressor T cells in acute IM and their subsequent disappearance in the convalescent state. This has important implications in the suppression of B cell outgrowth in IM which may be an important mechanism of containment of disease activity and prevention of evolution into a B cell neoplasm.

In addition, we have demonstrated the alteration of immunoregulatory T cell subsets as well as the presence of adherent suppressor monocytes in sarcoidosis and tuberculosis.

4) Because of the potential relationship between certain of the abovementioned autoimmune diseases and aberrancies of normally occurring autoreactivity, we investigated the mechanisms of regulation of autologous and allogeneic reactivity in the models of the autologous and allogeneic mixed lymphocyte reactions (MLR). In addition, we have defined the relative stimulatory and responder capabilities of various lymphocyte and monocyte subpopulations in the MLR. The relationship between the autologous and allogeneic MLR and the development of suppressor and/or cytotoxic T cells was described. In this regard, the presence of a potent adherent suppressor cell of the autologous MLR was demonstrated in sarcoidosis. Furthermore, the modulation of the autologous and allogeneic MLR in normal subjects by adherent cells, prostaglandins, irradiation and in vitro and in vivo corticosteroids has been delineated.

5) Because of the potential role of cytotoxic cells mediating either antibody dependent cellular cytotoxicity (ADCC) or natural killer (NK) activity in immunologically mediated disease, we have precisely delineated the ADCC and NK capabilities of multiple heterogeneous subpopulations of T cells, null cells and monocytes. In these studies, we have demonstrated the overlapping and distinct cytotoxic capabilities of these heterogeneous lympohoid cell subsets.

6) We have adapted a unique cell fractionation technique called elutriation (counter-current centrifugation) for the purpose of purifying human blood monocytes. This technique provides a major advance in our ability to carefully and precisely study the human monocyte.

This technique has the advantage over the current adherence techniques of monocyte isolation of negatively selecting the human monocyte in excellent purity (95%) and excellent yield (95%). The yield of this procedure is so great, that up to 1.5 billion monocytes have been purified from the blood of a single normal human at one time. In addition, this technique has proved to provide much more effective monocyte depletion of mononuclear cell suspensions than the current technique of depleting adherent cells on sephadex G-10 columns since elutriation does not remove non-monocyte adherent cells (such as B cells). Thus, we have been able to characterize large numbers of purified human monocytes with regard to their intracytoplasmic enzyme activity. We have also examined monocytes with scanning and transmission electron microscopy for the details of their cytoskeleton assembly. In addition, we have placed large numbers of these cells into culture to examine how these parameters are altered when these cells mature into macrophages. We have been able to examine the evolution of these functions on a daily basis for one week using a single normal individual's monocytes. Human monocytes have been examined for their ability to perform the interrelated functions of phagocytosis and killing in an ADCC system. We have shown that while monocytes have improved phagocytic function after being placed in culture, they have diminished killing capability in ADCC. Of interest is that we are the first group to demonstrate spontaneous killing of monocytes against human red cells and that this function improves when monocytes mature into macrophages. Hence, for the first time, human monocytes in a highly purified, non-activated state have been precisely characterized.

7) We have characterized functionally distinct subpopulations of T cells on the basis of relatively unstable surface receptors such as the Fc receptor for IgG or IgM (T_G or T_M). In addition, we have developed and characterized a rabbit anti-human T cell antiserum against stable cell surface components.

Most importantly, we are among the first few laboratories in the world to have produced and fully characterized a variety of mouse lymphocyte hybrid cell lines which are producing virtually unlimited quantities of antibodies which bind to a number of distinct as well as overlapping subpopulations of human peripheral blood mononuclear cells. In particular, antisera have been produced which bind to a functionally distinct human peripheral blood T cell subset. In addition, another antibody is being produced which binds specifically to human blood monocytes.

8) In line with our interest in the pharmocologic modulation of the immunoregulatory apparatus in man, we have studied and characterized the selective and differential effects of a number of agents directly on effector lymphocye subsets as well as on functionally distinct immunoregulatory lymphocyte subsets. These agents include corticosteroids, cyclophosphamide, azathioprine, irradiation, cyclic nucleotides, and prostaglandin inhibitors. A graded differential sensitivity of B cells, more than suppressor T cells, more than helper T cells was demonstrated to cyclophosphamide, azathioprine and irradiation. Cyclic AMP directly inhibited suppressor T cells, while prostaglandin inhibitors blocked prostaglandin mediated suppression of certain subsets of adherent suppressor cells. Corticosteroids had the most complex effects with selective and differential effects on the circulatory kinetics and functional capabilities of T and B cell subsets.

We have also delineated the relationship of corticosteroid receptors on lymphocyte subsets (with regard to receptor density, binding affinity and dissociation constant) and the differential effects of in vivo and in vitro corticosteroids on these subsets.

9) We have continued our clinical studies of a number of diseases of established or suspected immunologic mediation. These comprise virtually the entire spectrum of vasculitis including Wegener's granulomatosis, systemic necrotizing vasculitis (polyarteritis nodosa type), hypersensitivity vasculitis, Takayasu's arteritis, temporal arteritis, and lymphomatoid garnulomatosis. In addition, we are studing idiopathic midline granuloma, sarcoidosis, granulomatous hepatitis, juvenile rheumatoid arthritis, Weber-Christian panniculitis, a heterogeneous group of acquired immunodeficiency diseases, and host defense defects (chronic granulomatous disease, Chedick-Higashi syndrome), and a large number of patients with the idiopathic hypereosinophilic syndrome. The above patient groups are under our primary care. In addition, we are carrying on collaborative studies with others in SLE, Sjögren's syndrome, mixed connective tissue disease, rheumatoid arthritis, chronic lymphocytic leukemia, acute infectious mononucleosis, tuberculosis, and Cogan's syndrome.

In addition to carrying on extensive investigations delineating the mechanisms of altered immunologic reactivity in these diseases, the results of which are described above, we have established treatment protocols for several of these disorders. Major contributions have resulted from these and dramatic long-term remissions and even cures have been established by us in several of these formerly fatal diseases by the use of chronic low dose cytotoxic agents (usually cyclophosphmide), particularly in the severe systemic necrotizing vasulitides. These results are either recently published or in press. In particular, we continue to prospectively follow the largest group of patients with Wegener's granulomatosis in the world and have established a greater than 90% remission rate with the use of cyclophosphamide. In addition, we have most recently published similar striking results with cyclophosphamide in systemic necrotizing vasculitis of the polyarteritis nodosa group.

In addition, we are prospectively following the largest group of patients in the world with the idiopathic hypereosinophilic syndrome and have effected striking remissions by the use of hydroxyurea which has been shown to prevent and/or halt the eosinophilic myocardopathy which is the major source of morbidity and mortality in this disease.

Given the striking therapeutic results mentioned above, our group has been the major referral center for these diseases and the therapeutic protocols which we have established are now being adopted and employed successfully world-wide.

Proposed Course:

These projects will continue along the lines which have been described.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00056-06

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Biochemical Pathways of Mediator Release and Mechanism of Tissue Injury in Allergic Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A.P. Kaplan	Head, Allergic Diseases Section	LCI NIAID
OTHER:	M.A. Kaliner	Senior Investigator	LCI NIAID
	J.I. Gallin	Senior Investigator	LCI NIAID
	R.J. Mandle, Jr.	Guest Worker	LCI NIAID
	G.G. Miller	Clinical Associate	LCI NIAID
	H.L. Meier	Chemist	LCI NIAID
	R.E. Thompson	Chemist	LCI NIAID
	A.L. Weinstein	Guest Worker	LCI NIAID

COOPERATING UNITS (if any)

Z. Horakova	Senior Investigator	NHLI
S. Katz	Acting Director, Dermatology Branch	NCI
R. Sigler	Fellow, Walter Reed Army Hospital	

LAB/BRANCH
Laboratory of Clinical Investigation

SECTION
Allergic Diseases Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5 7/12	3 7/12	2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

INACTIVE DURING CURRENT YEAR. TERMINATED.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00057-06 LCI

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Basic Studies on Pathogenic Fungi

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K.J. Kwon-Chung	Senior Investigator	LCI NIAID
OTHER:	J.E. Bennett	Head, Clinical Mycology Sec.	" "
	Itzhack Polacheck	Visiting Fellow	" "

COOPERATING UNITS (if any)

A.K. Bhattacharjee (NIAMDD)

LAB/BRANCH

Laboratory of Clinical Investigation

SECTION

Clinical Mycology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	2	2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The interests and proposed course of our unit are to cover basic and applied aspects of various pathogenic fungi including their morphology, taxonomy, pathology, epidemiology, biochemistry and genetics. Topics of present interest include: 1) structure of the capsular polysaccharide of serotype D of Cryptococcus neoformans, 2) metabolic pathway of creatinine in Cryptococcus neoformans and C. bacillisporus, 3) nuclear status of basidiospores in the sexual state of C. neoformans, and 4) distribution of α and a mating types among clinical and natural isolates of C. neoformans and C. bacillisporus.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

Objectives of the project cover both basic and applied aspects of various pathogenic fungi, including their morphology, taxonomy, pathogenicity, life cycle, biochemistry and genetics. The topics of present interest include:

1) Chemical structure of capsular polysaccharide of serotype D of Cryptococcus neoformans.

2) Metabolic pathway of creatinine in C. neoformans and C. bacillisporus.

3) Nuclear status (cytogenetics) of basidiospores in the sexual state of C. neoformans.

4) Distribution of α and a mating types among clinical and natural isolates of C. neoformans and C. bacillisporus.

Methods Employed:

1) Growth and the Preparation of the Cell-free Extracts: Cells of C. neoformans and C. bacillisporus were grown on minimal medium containing creatinine or ammonium sulfate as the nitrogen source. The cells were harvested, washed and suspended in phosphate buffer (0.05 m), and broken by using glass beads and voltex mixture. The supernatant obtained after 10 min. breakage was used as cell-free extracts.

2) Metabolic Product of Creatinine: The cell-free extract was incubated with radiolabled creatinine. The radiolabled metabolites were detected by thin layer chromatography on silica gel with phenol-ethanol-water as the developing solution. The product was identified by autoradiographic technique.

3) Enzyme Assay: A very sensitive assay method was developed for creatinine deiminase by using the reaction mixture containing radiolabled creatinine buffer and cell-free extract. The enzyme reaction was stopped by adding acetic acid. The reaction mixture was shaken with cation exchanger Dowex-50 and then filtered. Under this condition, the substrate was found on the filter and the radioactivity of the filtrate represented the enzymatic product.

Genetic Analysis: Crosses of isolates with appropriate genetic markers were made on hay infusion agar. The progeny from such crosses were isolated by micromanipulation and the genetic markers were analyzed.

Production of Capsular Polysaccharide: Cultures were grown on Sabouraud dextrose broth, and cells were killed by adding buffered formalin

and removed by centrifugation. The supernatant was treated with sodium acetate, acetic acid and 95% ethanol to precipitate polysaccharide. The polysaccharide was dissolved in distilled water and treated with sodium acetate and acetic acid. The solution was deproteinated with chloroform and butanol. The aqueous solution was treated with sodium acetate, acetic acid and ethanol. The precipitate was redissolved in distilled water and reprecipitated with 95% ethanol. The polysaccharide was chromatographed on a column of DEAE-cellulose using 0.01 M phosphate buffer, pH 7.3, and a linear salt gradient of 0-1 M NaCl. The 80% of material was eluded at a salt conc. 0.2 M, and this material was dialized and freeze dried.

Chemical Structure of the Polysaccharide: Gel filtration, ultracentrifugation, paper chromatography, hydrolysis by 1 N HCl, methylation, periodate oxidation and chromium trioxide oxidation methods were used to analyze the chemical structure of the polysaccharide.

Major Findings and Significance to Biomedical Research and the Program of the Institute:

1) The most significant series of developments in this laboratory over the past year with important implications to biomedical research have been the series of studies which have shown that the etiologic agent of cryptococcosis is two distinct species of genus Cryptococcus rather than a single species, C. neoformans, as has been believed for the past 80 years. We have utilized genetical, biochemical, chemical and epidemiological aspects to reveal the distinct characteristics of the two species, C. neoformans and C. bacillisporus.

2) Significant Findings in the Area of Biochemistry:

a) The demonstration of two different regulatory mechanisms for the synthesis of creatinine deiminase between C. neoformans (serotype A-D) and C. bacillisporus (serotype B-C). One of the best known natural reservoirs of the etiologic agent of cryptococcosis is pigeon droppings. The widely accepted view is that pigeon droppings contain high concentration of creatinine, and it serves as a selective medium for the growth of cryptococcal pathogens. We reported in the past that only two serotypes (A,D) of Cryptococcus neoformans were found in the pigeon droppings throughout the world. Also reported was that serotype B-C utilized creatinine better than A-D and the natural reservoir of the B-C remained unknown. We described the serotype B-C as a distinct species, C. bacillisporus. Metabolic pathway of creatinine is known in several species of bacteria but not in fungi. Our study demonstrated that the creatinine metabolism in the cryptococci involved one step resulting in methylhydantoin and ammonia. The enzyme responsible for this degradation was identified as creatinine deiminase and was found to be inducible in both species. However, the enzyme synthesis was regulated by the presence of ammonia in C. neoformans but not in C. bacillisporus. This difference may be due to their ecological difference.

b) An extremely sensitive assay method for creatinine desimidase was developed during this study by using autoradiogram.

3) Findings in the Area of Genetics: We have further extended our previous studies on the cytogenetics of basidiospore formation in Filobasidiella neoformans (C. neoformans). Using two stable genetic markers, nuclear status of spore chains was clarified. Nuclear inclusion in the spore chains was found to be at random. Also demonstrated was the formation of diploid spores which go through meiosis during the blastospore formation.

4) Findings in the Area of Epidemiology: Survey revealed that the mating type α is predominant among natural and clinical isolates of Cryptococcus neoformans regardless of the serotype. The ratio of α and a type was about 40:1 among 105 natural isolates and 30:1 in 233 clinical isolates.

5) Findings in the Area of Immunochemistry: Capsular polysaccharide of Cryptococci contains antigenic determinants defining their serotypes. We extended the study on the chemical structure of serotype D of C. neoformans. The major sugars were found to be similar to that of C. bacillisporus but contained O-acetyl group which was not found in C. bacillisporus. Also found was that the substitution of mannose backbone by xylose and glucose was simpler than serotype A of C. neoformans and also isolates of C. bacillisporus.

Proposed Course:

The biochemical and genetical aspects of 5FC resistant strains and phenoloxidase negative strains of C. neoformans will be studied. The chemical structure of capsular polysaccharide of serotype B, C. bacillisporus, will be studied.

Publications:

1. Kwon-Chung, K.J. and Bennett, J.E.: Distribution of α and a mating types of Cryptococcus neoformans among natural and clinical isolates. Am. J. Epidemiol. 108:337-341, 1978.

2. Kwon-Chung, K.J., Bennett, J.E. and Theodore, T.S.: Cryptococcus bacillisporus sp. nov.: Serotype B-C of Cryptococcus neoformans. Int. J. Syst. Bacteriol. 28:616-620, 1978.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00058-06 LCI

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

The Pathogenesis and Chemotherapy of Herpesvirus Infections in Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. Dolin (until Jan 1, 1979)	Head, Medical Virology Section	LCI NIAID
	S.E. Straus (since Jan 1, 1979)	Senior Investigator, MVS	" "
Other:	R.Schooley	Clinical Associate	" "
	R. Berg	" "	" "

COOPERATING UNITS (if any)

P. Howley (LP, NCI) and R. Whitley (Cooperative Antiviral Study Group)

LAB/BRANCH

Laboratory of Clinical Investigation

SECTION

Medical Virology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1-1/2	1/2

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The pathogenesis and natural history of herpesvirus infections in man are being investigated. Patients are being identified, followed clinically and diagnosed definitively by virus isolation. Studies of patients with infectious mononucleosis have documented a variety of effects of the causative agent, the EB virus, upon the target B lymphocyte. The development and nature of T-cell mediated immune responses to the EBV infected B cells have been characterized. The results of initial trials of adenine arabinoside therapy of serious herpesvirus infections have been substantiated. The tools are being developed to study the biology and molecular epidemiology of varicella-zoster virus infection.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

1) To determine the pathogenesis and natural history of herpesvirus infections in both normal and immune compromised humans.

2) To evaluate the efficacy of antiviral agents in the treatment of human herpesvirus infection. These infections include both minor and serious diseases produced by herpes simplex types 1 and 2, varicella-zoster and EB virus.

Methods Employed:

1) Patients with suspected herpesvirus infections are referred to the Medical Virology Section from the Clinical Center, the National Naval Medical Center, local hospitals and practicing physicians. The patients are evaluated and appropriate specimens are collected for virus isolation and serologic testing.

2) Virus isolation is performed in primary or continuous human or simian cell lines. The viruses are identified by the appearance of typical cytopathic changes and typed by immunofluorescent procedures where required.

3) In selected cases, the viral DNA is purified, digested by restriction endonucleases and analyzed by agarose gel electrophoresis. This provides epidemiologic information regarding the transmission of the virus and may provide structural correlates of viral pathogenicity.

4) The clinical efficacy of adenine arabinoside, adenine arabinoside monophosphate, acycloguanosine and ribavirin is being investigated in the treatment of herpesvirus infections. This unit is collaborating in a NIAID-sponsored multicenter trial evaluating adenine arabinoside in the treatment of herpes simplex encephalitis, herpes zoster infection of the immune compromised host, and chronic mucocutaneous herpes simplex infection.

Major Findings:

1) EB Virus Infection.

a) Studies of cell-mediated immune responses in patients with infectious mononucleosis have progressed. T lymphocytes from patients with antibody to EBV are capable of suppressing lymphoblastoid transformation of EBV infected autologous lymphocytes. The acquisition of this suppressive capacity is absent early during primary clinical infection, develops after the first several days and remains present for life. This T-cell function is independent of humoral antibody and is not HLA restricted.

b) EBV has been found to be a potent in vitro polyclonal activator of B cell function as manifested by induction of a plaque forming cell response and production of intracytoplasmic antibody. The PFC response is not T cell dependent but may be modulated by helper and/or suppressor T cells when present.

c) Lymphocytes from patients with infectious mononucleosis are hyporesponsive to polyclonal stimulation by EBV.

d) Polyclonal stimulation of B cells requires successful infection by live EB virus. Not all virally infected B cells, however, produce immunoglobulin, suggesting that cellular control mechanisms may play a role in the expression of EBV induced B lymphocyte activation.

2) Clinical trials of systemically administered adenine arabinoside in herpesvirus infections.

a) Herpes simplex encephalitis: The results of the earlier small collaborative study wherein adenine arabinoside (ARA-A) was demonstrated to substantially reduce mortality in herpes simplex encephalitis have been confirmed. An additional 78 patients have been treated with ARA-A in the nationwide trial, with a fatality rate of 31%, quite comparable to the 28% incidence reported in the first study. Additional trials will be initiated within the next several months to compare two new promising agents, adenine arabinoside monophosphate (ARA-AMP) and acycloguanosine, with ARA-A.

b) Varicella-zoster infections: Patients will continue to be enrolled for the next few months into the collaborative trial of ARA-A for VZ infections of immunocompromised patients. The earlier preliminary trial suggested that the drug speeds clearing of virus from vesicles and healing of lesions. The present trial is aimed at defining whether treatment initiated within the first 72 hours of infection is capable of preventing dissemination of virus and/or subsequent postherpetic neuralgia.

3) Characterization of nucleic acid from varicella-zoster virus: VZV DNA purified from clinical isolates is digested with restriction endonucleases and analyzed by agarose gel electrophoresis. Highly labeled DNA will be prepared by nick translation and used in sensitive reassociation kinetic analyses to compare the DNA from different isolates.

Significance to Biomedical Research and the Program of the Institute:

Herpesviruses produce substantial morbidity and mortality each year. Normal adults and children suffer individual or recurrent infections caused by members of the herpesvirus family. These infections range from rather benign afflictions to those such as herpes simplex encephalitis which when untreated causes death in 60-70% of individuals. Immune compromised individuals, of which there are an ever increasing number and variety, are

especially susceptible to herpes infections. The above studies have expanded current knowledge of the natural history and pathogenesis of these infections. The therapeutic trials in which we collaborate have been the first to clearly demonstrate efficacy of antiviral compounds for these types of infections. These results have been encouraging and pave the way for future studies with even more potent antiviral drugs.

Proposed Course:

With the recent departure of the former principal investigator, the direction of this laboratory will undergo some changes. It is expected that a more molecular approach will be applied to the analysis of the herpesvirus infection with particular emphasis on herpes simplex and varicella zoster infections. The mechanism of virus latency and reactivation will be explored. Sensitive DNA reassociation analyses using highly labeled VZ DNA probes will be performed in an effort to detect the presence of viral sequences in the genome of tissues obtained at autopsy from patients with a known history of zoster infection.

Clinical trials with antiviral agents will continue. Within the next year, the NIAID sponsored collaborative study group will initiate trials of ARA-AMP and acycloguanosine. These agents hold particular promise because they are minimally toxic, are able to attain higher tissue levels and appear significantly more potent than ARA-A. In addition, this laboratory will attempt to initiate within the next several months a trial of topical therapy of herpes genital infection with ribavirin, another promising new agent. This compound has been found in uncontrolled use to be extremely effective in superficial herpes simplex infection. Its true merits must be defined by placebo controlled trials.

Publications:

1. Whitley, R.J., et al: Adenine arabinoside therapy of biopsy proved herpes simplex encephalitis. N. Engl. J. Med. 297:289, 1977.

2. Whitley, R.J., et al: Adenine arabinoside therapy of herpes zoster in the immunosuppressed: NIAID Collaborative Antiviral Study. N. Engl. J. Med. 294:1193, 1976.

3. Haynes, B.F., Schooley, R.T., Grouse, J.E., Payling-Wright, C.R., Dolin, R. and Fauci, A.S.: Characterization of thymus-derived lymphocyte subsets in acute Epstein-Barr virus-induced infectious mononucleosis. J. Immunol. 122:699-702, 1979.

4. Schooley, R.T., Haynes, B.F., Grouse, J.E., Payling-Wright, C.R., Fauci, A.S. and Dolin, R.: Quantitative assessment of suppression of Epstein-Barr virus induced B-lymphocyte outgrowth. Manuscript in preparation.

5. Schooley, R.T., Haynes, B.F., Grouse, J.E., Payling-Wright, C.R., Fauci, A.S. and Dolin, R.: Mechanism of EBV-induced B-lymphocyte activation. Manuscript in preparation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00154-04 LCI

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Immunologic, Neurophysiologic, Biochemical and Cellular Events in Immediate Hypersensitivity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Michael A. Kaliner	Senior Investigator	LCI	NIAID
OTHER:	J. Shelhamer	Clinical Associate	LCI	NIAID
	L. Steel	Staff Fellow	LCI	NIAID
	H. Oertel	Visiting Fellow	LCI	NIAID
	M. Donlon	Guest Worker	LCI	NIAID
	S. Wescott	Bio. Lab. Tech. (Biol.)	LCI	NIAID
	G. Myers	Bio. Lab. Tech. (Biol.)	LCI	NIAID
	P. Davis	Clinical Associate	PMB	NIAMD

COOPERATING UNITS (if any)
Walter Reed Army Medical Center (R. Evans, R. Summers, L. Smith and R. Sigler).

LAB/BRANCH
Laboratory of Clinical Investigation

SECTION
Allergic Diseases Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6 1/2	4 1/2	2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Human asthma and rhinitis involve the excessive secretions of mucus. Cultured human airways incorporate radiolabeled molecules permitting monitoring of mucous secretion. Human lung mucus has been characterized biochemically and the immunologic as well as neuropharmacologic control of mucous secretion defined. The site and control of human lung parenchyma versus airway prostaglandin production has been identified and the factors generated by anaphylaxis causing prostaglandin synthesis isolated. The histologic responses to rat mast cell granules have been characterized both in rat and monkey skin and the factors responsible for eliciting the inflammation isolated and characterized. The relationship between calcium influx and rat mast cell degranulation has been analyzed as has the response of mast cells to histamine stimulation. Finally, the neuropharmacologic responsiveness of subjects with asthma, allergic rhinitis and cystic fibrosis has been studied.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

The goals of the Allergic Diseases Section are to expand our knowledge of all aspects of immediate hypersensitivity reactions. Our particular expertise and focus are defining the biochemical events which accompany and control the triggering of allergic reactions, the neurophysiologic mechanisms which potentially modulate these events, the characterization of the mediators of anaphylaxis and identification of the responses of relevant target tissues to these mediators.

We are concentrating on three primary areas: 1) Employing human lung tissue, we study "asthma in a test tube" in order to understand the immunologic triggers, biochemical concomitants and neurophysiologic controls of mediator release; the identification of additional mediators of anaphylaxis and the study of mucous secretion. 2) Rat peritoneal mast cells are utilized as a pure source of effector cells which may be readily studied in vitro and may amplify as well as initiate new areas of interest in relationship to the human lung model and 3) Clinical allergic asthma is being evaluated in an extensive protocol designed to expand our understanding of the neurophysiologic mileau of allergic individuals as compared with cystic fibrosis and normal subjects.

Methods Employed:

1. Human lung model: Lungs removed, usually for cancer resection, almost always contain large amounts of normal, functioning lung tissue. We obtain these lungs in cooperation with the surgical and pathology departments of all the major hospitals in the Bethesda area. The lung tissue is washed, fragmented and replicated into 200 mg samples and subsequently incubated in dilutions of allergic serum for 2 hours at 37°C or 18 hours at 25°C. After this "sensitization" period, the serum is removed and the fragments challenged with allergen. The interaction of the allergen with tissue (mast cell) bound IgE induces the release of histamine, slow-reacting substance of anaphylaxis (SRS-A), eosinophil chemotactic factor of anaphylaxis (ECF-A), and prostaglandin E_1 and $F_{2\alpha}$ (PGE_1 and $PGF_{2\alpha}$). These mediators may be assayed on the isolated terminal portion of the guinea pig's ileum (histamine, SRS-A), chemotactic chambers (ECF-A) or by radioimmunoassay (PGE_1, $PGF_{2\alpha}$).

We have been determining alterations in the tissue levels of cyclic AMP and cyclic GMP after treating lung with various agonists in order to define the role these nucleotides play in modulating the release of mediators. Cyclic AMP may be measured by either a protein kinase binding assay or radioimmunoassay while cyclic GMP is determined by radioimmunoassay after acetylation or succinylation.

Mucous glycoproteins are monitored by incorporating ^{3}H-glucosamine into the mucous glands during an 18 hour incubation period. Secretions are dialyzed against 6M urea, filtered over sepharose 2B, and analyzed by ion

exchange chromatography. Mucous glycoproteins may also be hydrolyzed by $NaBH_4$ or be focused on a sucrose gradiant in the presence of ampholytes.

2. Rat mast cells: Rat peritoneal and pleural mast cells are obtained from freshly sacrificed male Sprague-Dawley rats by lavage of these cavities. The peritoneal cells include 5% mast cells (or approximately 500,000 to 1 million/animal). The cells are used either in mixed cell suspensions or after purification by centrifugation into albumin cushions. Mast cells granules are isolated with intact perigranular membranes by sonication and centrifugation through sucrose cushions. Granules free of membranes are obtained by osmotic lysis of mast cells.

^{45}Ca Calcium studies with rat mast cells involve incubation of cells with ^{45}Ca, followed by rapid centrifugation through silicone oil into water. Histamine released into the upper layer is assayed by an automated fluorometric assay while ^{45}Ca associated with the cells spun into the bottom layer is quantitated by scintillation.

3. Clinical asthma study: The methods employed in this study are covered in detail in the clinical protocol and include: a) measurement of cutaneous blood flow by ^{133}xenon disappearance; b) pupillary responses as measured by a binocular pupillometer; c) airway obstruction as measured by flow-volume curves with and without helium inhalation; d) serum cyclic nucleotide responses as measured after intravenous isoproterenol administration.

Major Findings:

1) Human Lung

a) The response of human and guinea pig peripheral lung preparations to histamine stimulation was compared with airway smooth muscle preparation from the same species. Histamine induced both PGE and $PGF_{2\alpha}$ from both parenchymal preparations, only PGE from human airways and both PGE and $PGF_{2\alpha}$ from guinea pig airways. In order to analyze the mechanism of histamine-induced prostaglandin synthesis, the responses to KCL at membrane depolarizing concentrations and the muscle stimulant carbachol were studied. Both guinea pig and human airway preparations were equivalently stimulated with KCL or carbachol while neither of these agents stimulated lung parenchyma. Therefore, histamine, through H-1 receptor stimulation, generates prostaglandin synthesis from lung by direct stimulation of parenchymal cells and by causing muscle contraction in the airways.

b) Human airways can be maintained in organ culture for at least 96 hours. The mucous secreting cells take up radiolabeled sugars, amino sugars, amino acids and sulfate and incorporate these labels into newly synthesized glycoproteins. The glycoproteins produced can be differentiated by size (fraction A is > 7,000,000 daltons while fraction B is 400,000 daltons) on gel filtration and carbohydrate: protein ratios (fraction A=70:30; fraction B=20:80; wt:wt). However, both molecules elute from anion exchange columns at the same salt concentration, each has an identical isoelectic focusing point, each has an identical constituent sugar composition, each has similar amino acids constituting the protein core and the size of the protein core of each molecule is the same. Thus, human airway submucosal glands synthesize two similar but distinct glycoproteins.

Employing quantitation of non-dialyzable glycoprotein radiolabeled with 3H-glucosamine to monitor mucous secretion, the influence of immunologic challenge as well as several neurohornomal agonists on mucous secretion was analyzed. Reversed and direct anaphylaxis of airways increased mucous secretion. As supernatants rich in the various mediators released during anaphylaxis were also effective in increasing mucous release, we determined which mediator was responsible. Histamine acting through H-2 receptor stimulation was found to cause mucous secretion, as did $PGF_{2\alpha}$, $PGF_{1\alpha}$, PGD_2 and PGA_2. PGE_2 and thromboxane B_2 were ineffective. Muscarinic stimulation of mucous glands by methacholine augmented mucous release as did alpha adrenergic stimulation. Beta adrenergic stimulation was ineffective. Therefore, several mediators of anaphylaxis (including histamine and several prostaglandins) and several neurophormones (cholinergic and alpha adrenergic agonists) are capable of increasing mucous secretion from human airways.

c) Prostaglandin formation by human lung after anaphylaxis may in part be attributed to histamine. However, about 50% of the prostaglandin formation is independent of histamine. Several factors released from human lung during anaphylaxis are able to cause guinea pig lung parenchymal preparations to produce thromboxane B_2, $PGF_{2\alpha}$ and PGE. We have characterized these factors and now recognize that they consist of two major components. The larger is retained on a DM5 filter (> 5000 MW), and separates on a Sephadex G-150 column into two factors with apparant molecular weights of >200,000 and 55000 daltons. The second component filters through DM5 but is retained by UM05 filters (MW = <5000 but >5000), filters on Sephadex G25 with an apparant molecular weight of 1500, adheres to DE52 in 0.001 of NH_4HCO_3, pH7.8, and elutes in one major peak at 0.1M NH_4HCO_3. Thus, human lung undergoing anaphylaxis generates at least three molecules in addition to histamine which are capable of causing prostaglandin synthesis. Further purification and biologic characterization of these molecules are underway.

2) Rat Mast Cells

Mast Cell granules injected into the skin of rats and monkeys were found to cause significant inflammatory reactions. Granules isolated with intact perigranular membranes caused a polymorphonuclear-rich infiltrate apparent in 2 hours which peaked by 8 hours. Subsequently, an intense mononuclear infiltrate replaced the PMN's and peaked at 24-48 hours. Granules isolated free of membranes and washed free of elutable mediators injected into skin caused a somewhat less pronounced polymorphonuclear infiltrate and a somewhat greater mononuclear infiltrate. The responsible factors were found primarily in close association with the granule matrix. In fact, granule preparations washed free of elutable mediators were found to have three separable factors capable of inducing these infiltrates. These factors may be separated on filters - the two larger factors are retained on a UM10 filter while the small factor filters through UM10 but is retained by a UM05 filter. The larger molecules have apparent molecular weights of 200,000 and 70000 daltons respectively on gel filtration through Sephadex G-200. The smaller molecule chromatographs on Sephadex G-25 with a apparent MW of 1250. The smaller molecule binds to both anion and cation exchange columns and elutes as one major peak in a salt gradient. The larger molecules may be precipitated

by 10% ethyl alcohol but not by $(NH_4)_2SO_4$. Therefore, there are three factors constituent within the mast cell granule which elicit delayed inflammatory responses in rat skin. Further characterization of the structure and biologic activity of these molecules is underway.

b) Initiation of rat mast cell secretion involves transmembrane movement of extracellular calcium. The equilibration with extracellular calcium in resting mast cells is extremely rapid, being completed within 60 sec at 37°C in the presence of 0.8 mM calcium. Analysis of calcium movement into the rat mast cell undergoing a secretory event reveals that uptake is increased within 5-10 sec, only 50% is removed by subsequent exposure to EGTA and the time course of calcium uptake continues after granule release is completed. Calcium uptake was initiated by phosphatidyl serine (PS) added to the bathing medium in the absence of histamine release. As PS augments antigen-induced histamine release, the calcium translocation observed may relate to this property. ATP (10 mM) inhibits mast cell degranulation and chelates calcium; at 1mM, ATP induces release and stimulates calcium uptake. ATP does not require hydrolysis to ADP or AMP for this response and there is insufficient ATP released by the mast cell to suggest that secreted ATP has any influence on the release reaction. Further characterization of the role which calcium plays in degranulation by mast cells is ongoing.

c) Rat mast cell cyclic AMP and cyclic GMP levels are important in so far as these levels determine the capacity of mast cells to undergo degranulation. We are completing an analysis of factors responsible for changes in mast cell cyclic nucleotides and have found that histamine fails to change the mast cell levels. This observation suggests that the mast cell which is a major synthesis and storage site for histamine is either desensitized to histamine or has no functional histamine receptor sites.

3) Clinical Studies

The autonomic responses of asthmatic subjects have been compared with those of subjects with allergic rhinitis and normal controls. Alpha adrenergic function was assessed by pupillometry and cutaneous vascular responses. In both instances, the subjects with asthma were significantly more sensitive than both allergic rhinitis and normal control subjects. Beta adrenergic function has been analyzed by the cardiovascular and cyclic AMP responses to intravenous isoproterenol in these subjects. Patients with either asthma or allergic rhinitis have significant impairment of both beta adrenergic responses as compared with controls. Parasympathetic function has been analyzed by pupillometry, sweat responses and bronchial challenge. Again, patients with either allergic rhinitis or asthma demonstrate the same degree of exaggerated responsiveness in comparison with normal controls. Therefore, asthmatics are selectively more responsive to alpha adrenergic stimulation while both asthmatics and subjects with allergic rhinitis have increased cholinergic and dimished beta adrenergic responses.

Subjects manifesting reproducibly positive allergic skin tests but who are asymptomatic have also been examined. These subjects who are "pre-allergic" have reduced beta adrenergic, hyper-responsive cholinergic and normal alpha adrenergic responses much as the subjects with allergic rhinitis. Taken together these data indicate that reduced beta adrenergic responsivity is associated with the atopic state as is excessive cholinergic responsiveness. However, excessive alpha adrenergic responses are seen only in asthmatics.

More recently, we have completed an examination of 11 young adult subjects with cystic fibrosis and 9 parents (obligate heterozygotes) of cystic fibrosis children. The CF subjects were significantly more sensitive than asthmatics in regards to both alpha adrenergic and cholinergic responsiveness and equally abnormal in regards to depressed beta adrenergic responses. Only 3 of the CF's were concomitantly allergic and these 3 were relatively less abnormal than the other 8. Therefore, the autonomic abnormalities uncovered may be found in diseases other than allergy and may relate to glandular (mucus) secretion rather than muscle spasm.

The obligate heterozygotes were, as a group, abnormal in both alpha adrenergic and cholinergic responses although much less so than the affected children. This observation suggests that the hyper-responsiveness is inherited as a primary defect rather than appearing as a secondary phenomena.

Significance to Biomedical Research and the Program of the Institute

Increased understanding of the mechanisms of immediate hypersensitivity and its controls may eventually allow improved therapy of the 44 million Americans with allergic rhinitis, the 9 million with asthma and the 22 million with urticaria. The model systems employed permit sophisticated analysis of many of the aspects involved in vivo. Unquestionably, our analysis of mucous secretion will enable us to approach clinical problems of bronchorrhea (asthma, cystic fibrosis, chronic bronchitis) with a greater understanding as well as permit in vitro analysis of new modalities of therapy.

The observation that mast cell granules may elicit delayed inflammatory responses may provide the mechanisms for explaining certain long-observed but hitherto poorly-understood clinical problems such as: the epithelial destruction accompanying asthma, the chronic inflammation associated with perennial rhinitis, the hyper-reactive airways disease occuring in asthma and delayed responses seen with allergy skin tests. Isolation of a low molecular weight molecule eliciting this late phase response implies potent chemotactic properties and should allow studies of the cellular mechanisms of chemotaxis employing a biologically-relevant molecule.

Characterization of prostaglandin generating factors elaborated by human tissues undergoing biologic processes (allergic responses) will permit identification of the role these factors play in a variety of diseases including asthma. Further, purification of these factors will permit a detailed analysis of the mechanisms of prostaglandin synthesis.

Finally, analysis of the autonomic responsiveness of allergic and cystic fibrosis patients enables us to approach these diseases with a unique appreciation of the capacity of neurophysiologic processes to modulate and complicate these diseases. Clearly the abnormalities uncovered have therapeutic implications in both the CF and asthma populations.

In summary, the Allergic Diseases Section is approaching immediate hypersensitivity at several levels with interests in both clinical and basic areas. This approach is providing a mileau in which results are rapidly transmitted from the laboratory bench to the clinic and from which a finer appreciation of disease processes is evolving. The direction in which the lab is headed should continue to translate basic observations to enhanced clinical practice.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 A1 00155-04 LCI

PERIOD COVERED
October 1, 1977 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Phagocyte Cell Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The mechanism of leukocyte activation by chemotactic factors has been studied using electrophysiology, fluorescent probe, surface charge and ultrastructural techniques.

Studies assessing the mechanism of modulating leukocyte locomotion indicate that limited secretion of specific granules, which accompanies chemotaxis, is associated with increased cell adhesiveness and increased availability of chemoattractant receptors. Vigorous exocytosis is associated with depressed chemotaxis, decreased availability of chemoattractant receptors, chemoattractant hydrolysis by secreted products and markedly increased cell adherence and aggregation. Human pyrogen has been shown to be a potent stimulator of neutrophil exocytosis and activation of the hexose monophosphate shunt.

Studies of the two populations of neutrophils we had identified previously indicate that during the neutropenia that follows in vivo endotoxin or hemodialysis, a subpopulation of neutrophils with poorly demonstrable Fc receptors is the predominant neutrophil left in the circulation. Clinical studies assessing the effect of pharmacologic agents on the neutrophil subpopulations in normal subjects, patients with recurrent infection and host defense defects are underway.

Project Description

Objectives:

1) Study the mechanism of leukocyte chemotaxis.

2) Study the phenomenon of deactivation of leukocyte chemotaxis and to relate this to the pathophysiology of leukocyte dysfunction syndromes.

3) Develop new techniques to quantitate the chemotaxis and the spreading of living phagocytes.

4) Study the role of cations in leukocyte activation with particular emphasis on the utilization of indirect probes of membrane potential to study the relationship between changes in membrane potential and initiation of neutrophil motility, phagocytosis, degranulation and superoxide generation.

5) Study neutrophil subpopulations in normal subjects and in patients with neutrophil dysfunction.

6) Study the mechanism of neutrophil margination and aggregation at capillary beds prior to cell migration into tissues.

7) Study the mechanism of mobilization and secretion of neutrophil lysosomal granules, study the relationship of the secretory process to neutrophil function and dysfunction and to study how neutrophil secretory products may influence homeostasis.

8) Characterize and define the mechanism of abnormal neutrophil chemotaxis following thermal injury.

9) Study the role of fibroblast secretory products in the inflammatory response.

10) Study the effects of pharmacologic agents on leukocyte function in vitro and in vivo.

11) Assess the chemotactic activity of histamine for eosinophils in vivo.

Methods Employed

Peripheral blood leukocytes were separated from heparinized whole blood by dextran sedimentation and Hypaque-Ficoll separation. Chemotaxis was evaluated using a radioassay employing ^{51}Cr labeled leukocytes and a double micropore filter system or a single filter measuring the distance migrated into the filter by the population of responding cells. Cell adherence was measured by quantitating the number of leukocytes adhering to plastic surfaces or to nylon wool columns. Phagocytosis, bactericidal capacity, nitroblue tetrazolium dye reduction, hexose monophosphate shunt activity and superoxide generation were

measured using standard techniques. PMN receptors for the chemoattractant f-met-leu-phe were quantitated as published previously.

The electrophysiology of cultivated human macrophages was studied using standard intracellular recording techniques. The fluorescent probe dipentyloxacarbocyanine and the radioisotope trimethylphosphonium were used as indirect probes of membrane potential changes in neutrophils. Leukocyte surface charge was measured with a Zeiss cytopherometer. Intracellular calcium was localized in human neutrophils by the intracellular precipitation of pyroantimonate anion followed by microprobe analysis and studies with the metal chelators (EDTA and EGTA).

The cytoskeleton of polymorphonuclear leukocytes was studied during conditions of chemotaxis and chemokinesis using 0.45 μm micropore filters. These small filters impede leukocyte migration but permit pseudopod penetration and a fixed orientation of leukocytes is thereby established. Orientation of living cells was monitored by well established techniques. Secretion of leukocyte granule enzymes were monitored by standard spectrophotometric assays. Polymorphonuclear leukocyte granules were separated and fractionated using sucrose gradient techniques and various enzyme markers were utilized to identify granule types.

Neutrophils were fractionated into subpopulations based on their ability to rosette IgG coated erythrocytes. In some studies, in normal human volunteers, the effects of intravenous endotoxin on neutrophil subpopulations was examined.

Major Findings

1. Technologic advances have been made for quantitating leukocyte chemotaxis, and bactericidal activity. Using an Optomax image analyzer the amount of time required to quantitate the number of cells migrating a distance into a micropore filter or the number of bacteria on a pour plate has been reduced by 90%. In addition, the image analyzer has made possible a quantitative assay of leukocyte spreading. Preliminary studies indicate the analyzer will make possible the tracking of moving cells thereby providing quantitative information on initial rates of locomotion (Alling).

2. During chemotaxis *in vitro* and exudation *in vivo* neutrophils secrete their granule contents and this secretion is preferential for specific granules. It has been shown that this secretory event modulates chemotactic responsiveness. With limited exocytosis there is increased chemoattractant binding to the cell membrane and this increased binding of chemoattractants increases availability of chemoattractant receptors without altered affinity of binding. Preliminary data indicates the increased receptor availability is related to translocation of specific granule membrane, which contains the chemoattractant receptors, to the cytoplasmic membrane. This may provide the mechanism for membrane turnover during sustained locomotion. Following vigorous exocytosis both chemotaxis and cell orientation in a gradient of chemoattractant are inhibited. This inhibition is related to decreased binding of a chemotactic factor to the cell. The latter has been associated with decreased peptide affinity and increased hydrolysis of chemoattractant by granule products secreted extracellu-

larly (Fletcher, Schiffmann).

3. Exocytosis of neutrophil granules *in vitro* increases neutrophil adhesiveness and aggregation. This has been related to neutralization of the net negative surface charge of the cell membrane and facilitation of cell-cell and cell-surface adhesion. Treatment of neutrophils with neuraminidase or poly-l-lysine also increases their adhesiveness without causing degranulation. In addition, submembranous deposition of cations (calcium) at the leading edge of cells in a gradient of chemoattractant was observed and speculated to facilitate fusion of intracellular granules with the cytoplasmic membrane. In this regard, it is of interest that the specific granules, which are most accessible to extracellular release, are more negatively charged than the azurophil granules (Gallin, Cramer).

4. Using fluorescent cyanine dyes and tritiated trimethyl phosphonium ions chemoattractants and degranulating stimuli were shown to stimulate membrane potential changes in neutrophils. The potential changes in neutrophils are similar to those observed in macrophages using direct intracellular recording techniques. The data using degranulating stimuli indicate low concentrations of degranulating stimuli increase f-met-leu-phe receptor efficacy for eliciting membrane potential changes as monitored with a fluorescent dye. Vigorous secretion inhibits receptor efficacy. These latter observations support and extend the data on the effect of degranulating stimuli on chemoattractant receptor availability and on chemotactic responsiveness. In addition, the data suggest that alteration of neutrophil plasma membrane ion permeability or the concentration of intracellular ions by degranulating stimuli modulates the locomotory responsiveness of the cell.

5. Assessment of chemoattractant elicited membrane potential changes using indirect probes indicates that neutrophils from patients with chronic granulomatous disease have a major abnormality. Neutrophils from other patients with chemotactic defects did not show any such abnormality (Seligmann).

6. Highly purified leukocyte pyrogen causes selective secretion of human neutrophil specific granules *in vitro* and rabbit neutrophils *in vivo*. In addition, pyrogen stimulates neutrophil nitroblue tetrazolium dye reduction, hexosemonophosphate shunt activation and superoxide generation (Klempner).

7. The acquired defect of neutrophil locomotion seen following thermal injury precedes sepsis and was shown to be a function of burn wound area and to be linearly related to the degree of lysosome lost from the neutrophil. A rabbit model for thermal injury has been developed to enable further study of this acquired chemotactic defect (Davis).

8. Twenty minutes following intravenous administration of *E. coli* endotoxin to normal volunteers, during the period of neutropenia, the predominant circulating neutrophil is a "subpopulation" without readily demonstrable IgG (Fc) receptors. Plasma obtained at the time of neutropenia increases neutrophil adhesiveness. In related studies, twenty minutes after initiating hemodialysis in patients with chronic Schizophrenia, also during a period of neutro-

penia, the remaining circulating neutrophils are significantly enriched with a subpopulation of neutrophils with poorly demonstable Fc receptors. Plasma obtained at this time during hemodialysis increased the adhesiveness and aggregation of control leukocytes (Klempner, Cotton).

9. Hydrocortisone sodium succinate reversibly inhibits adherence of IgG sensitized erythrocytes to human peripheral blood neutrophil monolayers suggesting hydrocortisone interferes with the availability of the neutrophil Fc receptor for binding (Klempner).

10. Normal human neutrophils were separated into two populations based on their ability to rosette human IgG coated erythrocytes and tested for their to rosette complement-coated erythrocytes. Both neutrophil populations formed rosettes with complement-coated erythrocytes equally well. Moreover, neither population of cells displayed those complement receptors felt to be markers of immature granulocytes (Whited, Frank).

11. Administration of prednisone to normal human subjects inhibits in vitro neutrophil and eosinophil adherence to nylon wool. In vivo prednisone also inhibits eosinophil but not neutrophil chemotaxis (Fauci).

12. Collection of human neutrophils by filtration leukapheresis for subsequent intravenous administration results in functional changes of the neutrophils attributable to degranulation and secretion of granule contents. Colchicine pretreatment of filtration leukapheresis donors significantly reduces these adherence induced changes (Wright).

13. Human fibroblasts cultured in vitro secrete at least two chemoattractants which, based on their elution from G-75 Sephadex chromatography columns, have molecular weights of greater than 150,000 and less than 5,000 daltons. These attractants appear distinct from collagen and other previsouly described chemotactic factors. They are protein in nature and attract both polymorphonuclear leukocytes and monocytes. Elaboration of this material in vitro is inhibited by colchicine (10^{-6} M) and hydrocortisone sodium succinate (1 mM) (Gallin).

14. In studies of patients with recurrent pyrogenic infections, a patient with abnormal neutrophil chemotaxis whose neutrophils are missing a membrane glycoprotein and impaired neutrophil spreading has been identified (Babior).

Significance to Biomedical Research and the Program of the Institute

The accumulation of leukocytes at inflammatory, immune and allergic sites is critical for appropriate responses. Understanding the physiologic basis for events regulating these processes will provide the basis for therapeutic manipulation.

One of the first steps in leukocyte mobilization from the blood stream is increased cell adhesiveness to the endothelium. This is followed by local

leukocyte aggregation and then diapedesis. Our finding that limited degranulation and exocytosis of intracellular granules markedly enhances these events, together with the observation that human pyrogen initiates these processes, provides a clue as to what controls margination and then migration of leukocytes from the blood stream. The related observation that vigorous degranulation in vitro inhibits chemotaxis suggested that some acquired chemotactic defects relate to excessive degranulation in vivo. In support of this is our finding that following thermal injury the severity of the acquired chemotactic defect is linearly related to the amount of intracellular lysozyme released. Clinically, it is of interest that the degranulation and defective chemotaxis precedes the severe and often lethal pyrogenic infections that follow thermal injury.

In related studies we confirmed that neutrophil specific granules contain lactoferrin and showed this is secreted when cells are incubated with pyrogen. Lactoferrin irreversibly binds iron which is then sequestered in the reticuloendothelial system. Another specific granule component, B_{12} binding protein, may effect B_{12} related events when secreted from the neutrophil. Thus, the potential regulatory role of neutrophil products on a number of systems is interesting and currently under investigation.

Two observations from our studies of the mechanism of the phenomena of degranulation, cell adhesiveness and chemotaxis may be particularly important to the cell biology of chemotaxis and perhaps relevant to clinical studies as well. We have shown that leukocyte adherence, aggregation and secretion may be under the modulating influence of electrostatic forces. Development of techniques for controlling these forces in vivo may have potential clinical use. Our data that neutrophil specific granules are a potential source of new cytoplasmic membrane and chemoattractant receptors is intriguing in terms of understanding the basis for membrane turnover during chemotaxis. These studies need to be extended and explored further.

The studies of the effect of steroids on PMNs are interesting and have shown that prednisone in vivo inhibits both neutrophil and eosinophil adherence and eosinophil chemotaxis. Hydrocortisone sodium succinate also blocks the expressability of neutrophil Fc receptors and inhibits chemotactic factor induced changes of neutrophil and macrophage membrane potential. These data contribute to our understanding of the physiologic basis for steroid action on these cells.

The use of fluorescent carbocyanine dyes to measure membrane potential is emerging as a rapid test of cell reponsiveness that appears to be as reliable and easier to use than other indirect probes of membrane potential. The demonstration of a severe abnormality of chronic granulomatous disease neutrophils using this test may provide a new simple rapid diagnostic test for this disease. In addition, the implications that the observed abnormality in chronic granulomatous disease reflects abnormal ion flux studies, perhaps related to abnormal activation mechanisms provides new insights into the nature of the defect.

The continuation of our studies of neutrophil subpopulations has indicated that one population of cell (PMNs with readily demonstrable Fc receptors) is

particularly available for margination. We are presently extending these studies of neutrophil subpopulations to explore their kinetics of circulation, and their functional role. In addition, the effect of various disease states and pharmcologic agents on these two neutrophil populations in man and in animals is under study.

Prosposed Course

We plan to continue some of the studies outlined above.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00188-01 LCI

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Rapid Diagnosis of Infections by Enzyme-Linked Immunoadsorbent Assays

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Medical Virology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Enzyme-linked immunoadsorbent assays have been developed to detect influenza A hemagglutinin, candida cell wall mannan, human adenoviruses, herpes simplex virus, and antibodies to pneumococci. Current efforts are designed to augment the sensitivity of the assays and establish their utility in clinical and research applications.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

1) To develop an assay which will rapidly, simply and reliably detect candida antigenemia, with the aim of identifying those patients who may benefit from early therapy with amphotericin B.

2) To develop a rapid assay for influenza infection in order to identify individuals and patient groups which may benefit from amantadine chemoprophylaxis.

3) To develop rapid, more sensitive assays to detect adenoviruses and herpes viruses in body fluids and in research specimens.

4) To develop a convenient assay for the development of antibodies directed against specific strains of pneumococci.

5) To detect group A streptococcal polysaccharide in throat swabs of patients with pharyngitis. The rapidity of the test would eliminate the 1-2 day wait for identification by routine microbiologic methods.

Methods Employed:

Either direct or competitive assays are employed. Briefly, the desired antigen is adsorbed to the surface of plastic microtiter wells. Specific antisera is added and binds to the antigen where present. Development of the antigen-antibody reaction is detected by addition of a second enzyme-linked antibody directed at the first antibody. The reaction is detected colorimetrically by addition of a chromogenic substrate specific for the enzyme.

Major Findings:

1) Candida: An ELISA has been developed which detects mannan at the nanogram level. A limited retrospective clinical trial has been completed. Briefly, sera of patients with proven disseminated candidiasis and those without candidiasis were tested in a blinded fashion. The assay discerned significant differences in level of candida mannan between the groups.

2) An ELISA capable of detecting less than 1 hemagglutinin unit of influenza A virus has been developed. The assay is currently being tested in experimental infections in ferrets.

3) Adenoviruses: An ELISA has been developed to reliably detect all of 26 types of human adenoviruses tested thus far. Modifications of the assay are being attempted which would permit virus typing.

4) Herpesviruses: ELISA tests for both types of herpes simplex are being developed using monoclonal type specific antisera.

5) Pneumococci: A successful assay is currently being tried in experimental pneumococcal infection of guinea pigs. A pilot study has been completed in which the ELISA was used to quantitate the antibody response to pneumococcal polyvalent vaccine in six volunteers. The assay results correlated very highly with results obtained by standard radioimmunoassay.

6) Streptococci: An assay using monoclonal specific antisera is being developed. The test thus far is insufficiently sensitive.

Significance to Biomedical Research and the Program of the Institute:

Rapid assay for a variety of antigens and antibodies has broad applicability to both clinical and research areas. The tests currently being developed will speed up, simplify, and reduce the cost of detecting infections. At the same time, they will permit clinical decisions regarding therapy to be soundly based at an earlier time in the course of an illness than is currently practiced.

Proposed Course:

Once the ELISA tests are fully developed and their sensitivity and specificity defined, they will be examined prospectively with patient material. Their ability to correctly identify infectious agents will be tested by comparison with the results of traditional methods. Assays for viral agents will also be applied to routine testing of research materials generated in the study of virus pathogenesis and treatment.

Publications:

1. Weiner, M.H., Yount, W.J.: Mannan antigenemia in the diagnosis of invasive candida infections. J. Clin. Invest. 58:1045, 1976.

2. Voller, A., et al: Microplate enzyme immunoassays for the immunodiagnosis of virus infections. In N.R. Rose and H. Friedman (eds.) Manual of Clinical Immunology. ASM, Washington, D.C., 1976, p 506.

3. Yolken, R.H., et al: Measurement of rotavirus antibody by an enzyme-linked immunoadsorbent assay blocking assay. J. Clin. Microb. 8:283, 1978.

4. Segal, E., Berg, R.A., et al: Detection of candida antigens in sera of patients with candidiasis by an ELISA test. J. Clin. Microb. In press.

5. Berg, R.A., et al: Type-specific pneumococcal antibody measurement with enzyme-linked immunoadsorbent assay. In preparation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00189-01 LCI

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Clinical and Biochemical Studies of Human Enteral Adenovirus Infections

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: S.E. Straus Senior Investigator
Medical Virology Section LCI NIAID

COOPERATING UNITS (if any)

A.Z. Kapikian (LID, NIAID)
H.S. Ginsberg (Columbia University)

LAB/BRANCH

Laboratory of Clinical Investigation

SECTION

Medical Virology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Enteral adenoviruses are a recently defined group of agents which cause gastroenteritis in children. They differ from other known human adenoviruses in their inability to be propagated in tissue culture. Adenoviruses recovered from patients will be examined for their relation to known respiratory adenoviruses by serologic, DNA hybridization, restriction endonuclease and protein electrophoresis studies. The mechanism of restriction of growth of these agents will be examined by nucleic acid hybridization, immunofluorescent microscopy and AAV helper studies. Attempts will be made to grow the viruses in tissue culture using adenovirus transformed cell lines and a series of early and late temperature sensitive mutants of adenovirus 5 as helpers. Enteral adenovirus-induced diarrheal illness will be studied in normal volunteers. The features of the illness, virus shedding and immune resolution will be examined.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

1) To define the clinical spectrum of illness induced by enteral adenoviruses in both naturally occurring and experimental infections.

2) To define the biochemical properties of these adenoviruses and their relation to other respiratory adenoviruses.

3) To develop methods of promoting the growth of these agents in tissue culture so that further studies will be simplified.

Methods Employed:

1) Source of viral agents: Enteral adenoviruses will be obtained from specimens collected during natural infections at Children's Hospital and identified as enteral adenoviruses by their EM appearance, reactivity in ELISA tests, and their fastidious behavior in standard tissue culture lines.

2) In vitro culture system: Standard primary and continuous human and simian, adult and fetal tissues will be employed for attempted virus isolation. A variety of methods will be employed to promote the efficient replication of these viruses in tissue culture. Stool specimens will be inoculated into an adenovirus transformed cell line (293) with the hope that the integrated viral sequences will complement the presumably restricted functions of the enteral viruses. Similar complementation studies will be attempted using early and late temperature-sensitive mutants of adenovirus type 5 which have been developed at Columbia University. Simian cells will be coinfected with SV40 and enteral adenoviruses to determine whether the helper function that SV40 provides for respiratory adenoviruses in monkey cells extends to these agents as well.

3) Biochemical Studies: The mechanism of restricted growth in tissue culture will be examined by performing DNA-DNA and DNA-RNA hybridizations on infected cell extracts using in vitro labeled DNA probes. Immunofluorescence microscopy will indicate whether adenovirus T antigen or late proteins are being synthesized in the restricted system. The expression of early adenovirus function will be assessed by testing for helper activity for adeno-associated viruses.

4) Clinical Studies: After rigorous safety testing, enteral adenovirus-containing stool filtrates will be innoculated into normal adult volunteers. The ability of these agents to reproduce the typically mild diarrheal illness will be explored. The illness will be characterized by close clinical observation, virologic and serologic studies of blood, stool and respiratory secretions, and electron microscopic examination of stools.

Major Findings:

Our laboratory has to date developed the tools necessary to perform these studies. We are now proficient with the maintenance of the required cell lines, the growth and replication of respiratory adenoviruses, labeling and extraction of viral nucleic acids and proteins, nucleic acid hybridization, restriction endonuclease analysis, SDS-PAGE, immunofluorescent microscopy, HA, HI, neutralization and ELISA tests. We are currently awaiting our first stool specimen.

Proposed Course:

Detailed investigation of the viral pathogenesis and host defense mechanisms will depend upon our ability to generate substantial quantities of virus and reproduce the clinical illness in volunteers. Since the other major viral diarrhea agents (rotaviruses, parvovirus-like agents) can not be grown in the laboratory, our success with these enteral adenoviruses may provide the model for the study of human viral gastroenteritis.

Publications:

1. Flewett, T.H., et al.: Epidemic viral enteritis in a long-stay children's ward. Lancet 1:4, 1975.

2. Richmond, S.J., et al.: An outbreak of gastroenteritis in young children caused by adenoviruses. Lancet 1:1178, 1979.

3. Dolin, R.: Viral infections of the gastrointestinal tract. In G.J. Galasso, et al. (eds.) Antiviral Agents and Viral Diseases. Raven Press, New York, N. Y., 1979, p. 289.

4. Wadell, G.: Classification of human adenoviruses by SDS-polyacrylamide gel electrophoresis of structural polypeptides. Intervirology 11:47, 1979.

LABORATORY OF IMMUNOGENETICS
1979 Annual Report
Table of Contents

Annual Report of the Laboratory of mmunogenetics, National Institute of Allergy and Infectious Diseases, NIH, October 1, 1978 - September 30, 1979

Summary Report

The Laboratory of Immunogenetics continues to conduct research aimed at elucidation of the genetic basis of immunologic function. Studies on genetic markers of immunoglobulins have concerned the expression of latent allotypes. In the past year progress has been made on structural determination of molecules bearing latent allotypes and more recently, interspecies cell hybrids (mouse-rabbit) are being utilized as a source of mRNA which will be used to initiate molecular biological studies which hope to elucidate the genetic basis for latent allotypes. Studies on the structure of histocompatibility molecules continue; the 280 residue papain fragment of the murine K^b molecule is near completion and intense structural analysis of molecules from this and other mouse MHC haplotypes have begun. In additon, progress has been made on studies of rabbit histocompatibility antigens this year. Other areas of active interest include studies of cell surface molecules using hybridoma reagents which have been applied with some effectiveness in our H-2 studies. Initially the hybridoma antisera will be directed against T-cells from the rabbit. Work on the control of the immune response by T-cell components has recently been initiated and this will involve a chemical and biological approach to this problem.

Recent additions to the LIG staff include Dr. Lee Maloy a Biochemist from Case Western Reserve University and Dr. Thomas Folks who joins us from the Naval Medical Research Unit in Bethesda. Dr. John Sogn has been given the position of Chemist, GS-13 after serving a year and one-half as Senior Staff Fellow in the Laboratory. Dr. Blair Fraser, a Staff Fellow left to accept a permanent position with the Bureau of Biologics. In addition to the permanent personnel our program has been greatly enriched by participation of students from various part-time and summer programs.

Research Accomplishments

Rabbit Histocompatibility Antigens. Products of the rabbit histocompatibility genes were isolated from a rabbit lymphoid tumor cell line (RL-5) following growth in culture with radioactive amino acids. Molecules were obtained from detergent lysates and glycoprotein fractions were isolated by affinity chromatography using immobilized lentil lectin. Chromatography on purified sheep anti-rabbit beta-2-microglobulin yielded a 43,000 dalton fraction which was noncovalently associated with beta-2-microglobulin. Amino terminal sequence analysis allowed assignment of 28 of the amino terminal 35 residues. These data revealed 89% homology between this RLA-11 product and the human HLA-b7 molecule. Similar comparison with the mouse $H\text{-}2K^b$ histocompatibility antigen yielded a homology of 82%. Although, beta-2-microglobulin is associated with histocompatibility antigens of several types (K,D, and L in the mouse) in other species only a single molecular species could be detected in our studies even though beta-2-microglobulin was the basis upon which our molecule was isolated. The extent of similarity between the rabbit and other histocompatibility cmplexes is currently under investigation.

Mass Spectrometric Analysis of Peptides. Immunogenetic studies require fast, reproducible and sensitive methods for amino acid sequence analysis of proteins which are not intrinsically radiolabelled. One possibility to attain this goal is mass spectrometric-gas chromatograhic (MS-GC) techniques. Work in the past year has included studies on a method which involves digestion of polypeptides into dipeptides from the amino terminus and identification of these dipeptides by computer aided MS methodology. More recently, the use of an enzyme dipeptidyl carboypeptidase (DCP), has been investigated for use with these techniques. This enzyme cleaves dipeptides from the carboxy terminus. It is anticipated that this enzyme will be extremely useful either as an adjunct to the DAP digestions, or as a method to complement normal Edman degradation. Model compounds under study at present include rabbit and mouse Ig heavy chains as well as rat beta-2-microglobulin.

Murine Transplantation Antigens. Gene products from murine major histocompatibility locus are involved in diverse functions which are associated with immune recognition and reaction. Our structural studies thus far, have dealt with classical transplantation antigens encoded at the K and D loci. Studies using radiochemical methodology have allowed assignment of nearly 280 residues to the K^b glycoprotein molecule. Data indicate a high degree of homology to human histocompatibility antigens and furthermore show that the differences between these molecules are not spread over the molecule but rather cluster in discrete areas. In addition to the K^b molecule, primary structural analysis is being carried out on the D^b, D^d and L^b molecules. In addition, the determination of the primary structure of murine beta-2-microglobulin, a molecule found in association with major histocompatibility antigens, is near completion. It is anticipated that knowledge of the primary structure of these molecules will aid in an understanding of their function and mechanism of action. The major histocompatibility antigens are deeply involved in allograft rejection and in the response of T lymphocytes to altered cell surface antigens.

Precursors of Immunoglobulin Molecules. When certain protein products are formed by *in vitro* translation using mRNA the products yielded are larger than those found for the normal secreted products. The amino terminus of certain of these proteins may be extended by as many as 30 amino acids. These precursor sequences have been reported for immunoglobulin light and heavy chains as well as a number of hormones and secreted enzymes. The present studies are directed toward a study of the immunoglobulin precursor molecules of the rabbit. This study will attempt to correlate the variable region extension with the constant region allotypes of the rabbit L chains. The source of mRNA, which in the past has proved troublesome, will be hybrid cell lines which secrete a single heavy or light chain of the rabbit.

Rabbit Latent Allotypes. Rabbit immunoglobulin allotypes are antigenic determinants which were thought to be inherited as autosomal codominant allele. This notion has been challenged by observations of low concentrations of allotypes which were not detected by qualitative tests

nor predicted by parental genotypes. Detailed structural analyses have shown that latent allotypes isolated from the sera of pedigreed rabbits are indistinguishable from the normal allotypes. Current research focuses on hybridoma cells maintained in culture that are able to synthesize chains with rabbit allotypes. RNA which encodes the immunoglobulin chains secreted by these cells will be isolated and cDNA probes will be prepared in order to carry out molecular biological studies aimed at elucidation of the basis for inheritance of latent allotypes. Other studies in this area have included measurement of the clearance of normal and latent allotypes in animals using doubly radiolabelled material. Other genetic studies in this area have concentrated on the linked expression of V_H markers of immunoglobulin expressing latent allotypes.

Honors and Awards

Dr. Kindt serves on the editorial boards of the Journal of Immunology, Proceedings of the Society for Experimental Biology and Medicine the Zeitschrift fur Immunotatsforschung, the Journal of Experimental Medicine and Molecular Immunology. He serves as a member of the program committee of the American Association of Immunologists and was elected this year to the American Society for Biological Chemists. Dr. Kindt was invited to speak at the Josiah Macy Foundation Conference on membranes and human disease which was held in New Orleans and the Institute Pasteur Conference on allotypes and idiotypes which was held in Paris. Seminars of laboratory work were presented by Dr. Kindt at the University of North Carolina at Chapel Hill, at New York University Medical School in New York, at Scripps Clinic and Research Foundation in La Jolla, at the Molecular Immunology Institute at the University of Marseille, at the Institute for Genetics at the University of Köln and at the German Cancer Research Institute in Heidelberg.

Dr. Coligan presented his recent data on the structure of mouse histocompatibility antigens in seminars at the Mayo Clinic in Rochester, Minnesota and at Harvard University. He also presented data at a symposium on T and B lymphocytes held at Keystone, Colorado and at the International Congress of Biochemistry in Toronto, Canada.

Martin Yarmush received his Ph.D. degree from Rockefeller University this Spring and was accepted at a number of medical schools. He will attend Yale University Medical School in the Fall.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00166-02 LIG

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Chemical Characterization of Rabbit Histocompatibility Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Thomas J Kindt	Chief, LIG	LIG	NIAID
Others:	Dr. Edward S. Kimball	Staff Fellow	LIG	NIAID
	Dr. John E. Coligan	Research Microbiologist	LIG	NIAID
	Dr. Frederick T. Gates	Staff Fellow	LIG	NIAID

COOPERATING UNITS (if any)
Dept. of Genetics, University of Illinois at Chicago (Dr. R. Tissot)

LAB/BRANCH
Laboratory of Immunogenetics

SECTION
Immunogenetics Research Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.9	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX(c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This laboratory has been engaged in the isolation and purification of histocompatibility antigens of the rabbit. There are approximately 12 serologically defined haplotypes in the domestic rabbit and our study has concentrated on the RLA 11 haplotype. The major source of antigen has been a tumor cell line derived from the Bar Harbor B strain rabbit. Antigen isolation was carried out by detergent solubilization of cells labelled with tritiated amino acids. Partial amino acids sequence analysis was carried out on the histocompatibility antigen isolated and it revealed a high degree of homology especially to histocompatiblity antigens isolated from humans. Beta-2 microglobulin, a low molecular weight protein that is associated with histocompatibility antigens, was subjected to total amino acid sequence analysis. Although this protein is homologous to analogs from other species some significant differences were observed in its structure.

PHS-6040
(Rev. 10-76)

Chemical Characterization of Rabbit Histocompatibility Antigens

Graft rejection and control of the ability to respond to specific antigens are directed by products of genes residing in the major histocompatibility complex. Little is known about the structure and function of the major histocompatibility gene products although preliminary work has been reported for proteins from the mouse and human. It is our intention to study the rabbit as a third species in order to compare MHC gene products structures. There are about twelve serologically defined MHC haplotypes in the domestic rabbit.

Current work has concentrated on the RLA-11 haplotype. The major source of antigen has been a tumor cell line derived from the Bar Harbor B strain of rabbits. Antigen isolation has been carried out by detergent solubilization of cell membranes that were intrinsically labeled with tritiated amino acids. A glycoprotein fraction was isolated by lentil lectin chromatography and the histocompatibility antigen isolated from this by affinity chromatograhy on a column of highly purified antibodies directed against beta-2 microglobulins. Amino terminal amino acid sequence analysis using radiochemical methods allowed the assignment of 28 of the amino terminal 35 residues. The data obtained revealed 89% homology of the RLA-11 protein with the human HLA B7 and 82% with the mouse H-$2K^{D}$. Comparison of the RLA-11 to sequence data obtained from major histocompatibility antigens of other species revealed no substitutions unique to the rabbit antigen.

The complete amino acid sequence of rabbit beta-2 microglobulin has been determined and compared to the sequence reported for human beta-2 microglobulin. This comparison showed a homology of 71% with a minimum of 13% difference in nucleotide sequences of genes encoding the two proteins. However, a single insertion must be introduced before position 68 of the rabbit protein to maintain this maximum homology. Amino acid substitutions are distributed throughout the molecule. Although the majority of those requiring multiple base changes are restricted to the carboxy-terminal third of the molecule. Although the rabbit protein analyzed in this study was isolated from the pooled urine of 15 rabbits no heterogeneity in amino acid sequence was observed.

Publications

Kimball, E.S., Coligan, J.E., and Kindt, T.J.: Structural characterization of antigens encoded by rabbit RLA-11 histocompatibility genes. Immunogenetics 8: 201-211, 1979.

Kindt, T.J., Coligan, J.E., Kimball, E.S., Ewenstein, B., Uehara, H., Martinko, J., and Nathenson, S.G.: Use of radiochemical techniques for primary structural analysis of mouse and rabbit histocompatibility antigens. Proceedings of the Josiah Macy Foundation, in press, 1979.

Gates, III, F.T., Coligan, J.E., and Kindt, T.J.: The complete amino acid sequence of rabbit beta-2-microglobulin. Biochemistry 18: 2267-2272, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00167-02 LIG

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Mass Spectrometry and Sequence Analysis of Polypeptides

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Henry C. Krutzsch	Sr. Staff Fellow	LIG	NIAID
Others:	Dr. Thomas J. Kindt	Chief, LIG	LIG	NIAID

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Immunogenetics

SECTION
Immunogenetics Research Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.9	0.9	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A major need in immunogenetic studies are fast, reproducible and sensitive methods for amino acid sequence analysis of proteins. Mass spectrometric (MS) techniques provide a promising means of meeting these goals. Methods under study involve enzymatic digestion of polypeptides into dipeptides using either dipeptidyl aminopeptidase or dipeptidyl carboxypeptidase (DAP/DCP method)and use of MS for rapid identification of dipeptides produced. Another employs direct introduction of larger derivatized peptides into the MS for analysis (DI method). Computer assisted data analysis programs for both MS methods are under development.

The DAP/DCP and DI methods are being tested using rabbit and mouse heavy chains as model proteins because these proteins are not readily amenable to routine sequence analysis techniques. The structure of rat β-2 microglobulin is also under study using this method.

PHS-6040
(Rev. 10-76)

Mass Spectrometry and Sequence Analysis of Polypeptides

It is the aim of this program to develop rapid and sensitive methods for polypeptide sequence analysis and to apply these methods to molecules of immunogenetic interest. Development of this method will include devising strategies for isolaton and purification of peptides in amounts sufficient for characterization. These techniques will facilitate polyeptide sequence analysis, helping to determine more rapidly the primary structures of immunogenetically important molecules such as antibodies and histocompatibility antigens.

An LKB Model 2091 EI-CI gas chromatograph-mass spectrometer (GC-MS) was used to identify trimethylsilylated (TMS) dipeptides from dipeptidyl peptidase (DP) digestion of polypeptides. Two MS ions are necessary for identification; the sequence-determining ion resulting from loss of the C-terminal residue plus the N-terminal residue's carbonyl group, and the molecular weight-determining ion. Alignment of the dipeptides to give the polypeptide sequence is done by using the dipeptides from a second DP digestion of the polypeptide after addition of another amino acid residue, or by homology to a similar peptide of known structure.

Some more areas of the DP/GC-MS method have been investigated. The utilization of dipeptidyl carbonsypeptidase (DCP), in this case Angiotensin-I converting enzyme, in place of DAP for sequence analysis from the C-terminus (instead of the N-terminus) of the polypeptide has been one of these. Use of this type of enzyme system will provide sequence information that may not be available from Edman chemistry (or DAP digestion) due to a blocked N-terminus, or to inability to go completely to the C-terminus in peptide with an unblocked N-terminus. At the present time, no totally reliable technology exists for such an operation.

In this area, three sections were investigated; enzymology, scope of digestion, including available sensitivity and associated chemistry. The enzymology was studied to determine what enzyme concentration, buffer conditions and associated miscellaneous conditions, such as digestion times, which gave maximum dipeptide yields. The enzyme system was also checked for the presence of materials that would interfere with the subsequent GC MS analysis, such as contaminating proteases or components that would create spurious GC peaks. The scope of the digestion was studied to determine what types and lengths of polypeptides could be digested, as well as the amount, in nmoles, of polypeptide that could be determined. The chemical studies were mainly concerned with determining and optimizing (and carrying out) the best method for obtaining the "overlapping" peptide to provide the necessary alignment information for the dipeptides from DCP digestion of the native polypeptide.

The results obtained up to the present indicate that the method has broad applicability. Thus, this system has been applied to peptides containing all types of amino acids, with chain lengths of up to greater than 50 residues, and at levels less than 5 nmoles.

Four computer programs, written in Fortran-IV, have been almost completely developed to allow rapid manipulation of the GC-MS DP digestion data. Two of these programs are for dipeptide identification, one a manual system, one an automatic system. The other two are for dipeptide alignment, either from the N-terminus (DAP digestion) or from the C-terminus (DCP digestion).

Two proteins are currently under study with these enzyme systems; the N-terminal 225 residue fragment resulting from CNBr cleavage of a rabbit (#4135, a3 allotype) homogeneous antibody heavy chain, and rat β-2 microglobulin. All of the work up to the present time has been with the DAP system, using as substrates peptides from both restricted (Arg cleavage only) and total tryptic cleavage of the rabbit H-chain CNBr fragment. Several partial or total sequences have been obtained for these peptides. Because several of these tryptic fragments are blocked at the N-terminus due to glutamine-pyroglutamate coversion, the DCP enzyme will be a useful addition to the present technology.

Publications

Krutzsch, H.C. and Pisano, J. Preparation of dipeptidyl aminopeptidase IV for polypeptide sequencing. Biochem. Biophys. Acta. 576: 280-289, 1979.

Krutzsch, H.C. and Kindt, T.J. The identification of trimethylsilylated dipeptides with chemical ionization mass spectrometry. Analytical Biochemistry 92: 525-531, 1979.

Rao, D.N., Rudikoff, S., Krutzsch, H., and Potter, M. Structural evidence for independent joining region gene in immunoglobulin heavy chains from anti-galactan myeloma proteins and its potential role in generating diversity in complementary-determining regions. Proc. Nat. Acad. Sci. 76: 2890-2894, 1979.

Keeton, T.K., Krutzsch, H.C., and Lovenberg, W. A specific radioimmuneassay for 3-methoxy-4-hydroxyphenyl-ethylene glycol (mopeg). Proc. 4th International Catecholamine Symposium., Pergamon Press, 1978.

Yarmush, M.L., Krutzsch, H.C., and Kindt, T.J. Amino acid sequence analysis of immunoglobulin light cains by gas chromatographic-mass spectrometric techniques: structural identity of latent b9 and nominal b9 molecules. Molecular Immunology, in press, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00168-02 LIG

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Structure of Rabbit Immunoglobulin and Antibody Heavy and Light Chains

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Thomas J. Kindt	Chief	LIG	NIAID
Others:	Dr. John A. Sogn	Chemist	LIG	NIAID
	Dr. Henry Krutzsch	Sr. Staff Fellow	LIG	NIAID

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Immunogenetics

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NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.3	0.7

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Homogeneous rabbit antibodies to streptococcal carbohydrates, are used to investigate structural aspects of the allotypic and idiotypic markers of immunoglobulins. The partial amino acid sequence was determined for a rabbit light chain bearing latent b9 allotype. In addition, structural work on a rabbit heavy chain bearing the a3 allotype is presently under way.

Studies such as these that combine serological and structural investigations of antibodies have the potential to define genetic mechanisms involved in antibody synthesis.

PHS-6040
(Rev. 10-76)

Structure of Rabbit Immunoglobulin and Antibody Heavy and Light Chains

The structural aspects of serologically detected polymorphic forms of rabbit immunoglobulins will aid in understanding the genetic mechanisms involved in antibody synthesis. Study of the covalent structure of immunoglobulins involves both separation of the protein into component peptides and amino acid sequence analysis of these materials. Separation of heavy and light chais from rabbit IgG is a standard procedure, but structural analysis of the components must be treated in a unique manner depending on the chain and its allotypic specificities. During the past year we have been determining structural features of light and heavy chains representing various allotypes.

In the area of light chain structure, the amino acid sequence for a latent b9 allotype light chain was determined, using the DAP/GC-MS method for residues 110-118, following mild acid cleavage and full reduction and alkylation of the whole isolated light chain. Comparison with similarly treated and DAP/GC-MS analyzed nominal b9 allotype isolated light chain showed the same sets of dipeptides, indicating that they are structurally, (latent and nominal b9 allotype) as well as serologically, identical.

In the area of heavy chain structure, work has been concentrated on determining the amino acid sequence for the N-terminal 225 residue piece from CNBr cleavage of heavy chain of allotype a3, isolated from homogeneous rabbit anti-streptococcal antibody. This fragment has been fully reduced and alkylated and subjected to partial and full tryptic cleavage. The amino acid sequence determination of these tryptic peptides is currently under way; both the conventinal Edman method and the DAP-DCP/GC-MS method are being employed. The structural studies on this CNBr fragment will serve as a prototype for future structural work on other proteins and peptides. New methods of peptide isolation and detection have been developed and are currently being applied to obtain peptides from chains such as this to allow complete structural analysis.

Publications

Yarmush, M.L., Krutzsch, H.C., and Kindt, T.J.: Amino acid sequence analysis of immunoglobulin light chains by gas chromatographic-mass spectrometric techniques: Structural identity of nominal and latent b9 molecules. Molecular Immunology, in press, 1979.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00169-02 LIG

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Primary Structural Analysis of Murine Transplantation Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Dr. W. Lee Maloy	Staff Fellow	LIG	NIAID
	Dr. Edward S. Kimball	Staff Fellow	LIG	NIAID

COOPERATING UNITS (if any)
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National Cancer Institute, National Institutes of Health (Dr. David Sachs and Dr. Ted Hansen)

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Laboratory of Immunogenetics, NIAID

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Immunogenetics Research Section

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	2.7	1.8

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The genes in the mouse H-2 major histocompatibility complex determine a diversity of functions associated with immune recognition and reaction. Three of these genes, H-2K, H-2D and H-2L encode the major antigens involved in allograft rejection; a feature undoubtedly related to the genetic polymorphism of these H-2 molecules. In addition, these molecules play a role in T-lymphocyte responses to altered cell-surface antigens. These H-2 molecules are integral membrane glycoproteins and are associated on the cell surface with β_2-microglobulin. The purpose of this work is to determine the primary structure of these molecules in order that we may better understand their function and mechanism of action. Toward this goal, we are determining the primary structure of each of these molecules from mice of the b and d haplotypes (i.e. H-2K^b, H-2D^b, H-2D^d, H-2L^d). The amino acid sequence analysis of H-2K^b is near completion and limited amounts of sequence data are available for the other molecules. In addition, the determination of the primary structure of murine β_2-microglobulin is near completion. 21-13

Primary Structural Analysis of Murine Transplantation Antigens

In order to relate structural differences among mouse H-2 glycoproteins to changes in their biological activity and to examine various genetic and evolutionary relationships among the genes of the major histocompatibility complex, the complete amino acid sequences of the H-2K and H-2D glycoproteins are being determined.

Because of the limited amounts of these materials available, radio-chemical methodology has been developed in order to pursue these studies. H-2 molecules are intrinsically radiolabeled by growing appropriate tumor cell lines in the presence of radioactive amino acids. Initial studies have utilized the EL4.BU ($H\text{-}2^b$) and C14 ($H\text{-}2^d$) lymphoblastoid cell lines. Following detergent extraction, the H-2 glycoproteins are isolated by immunoprecipitation with alloantiserum. Peptide fragments are produced by CNBr cleavage at methionine residues and are isolated by gel filtration and ion exchange chromatographic procedures.

Microsequencing analyses utilizing radiochemical methodology have made possible the complete primary structural determination of the $H\text{-}2K^b$ (H-2.33) molecule. Approximately 95% of the amino acid sequence of the NH_2-terminal 270 residues have been assigned. This portion of the molecule is roughly equivalent to the papain fragment and accounts for the extra cellular portion of the molecule. Amino acid sequence determinations of the transmembrane (30 residues) and the intracellular (40 residues) portions of the molecule are approximately 50% complete.

Comparison of the amino acid sequences for the $H\text{-}2K^b$ molecule and the human HLA-B7 molecule in regions currently available for comparision indicate an overall homology of 70%. Greater than 90% of the amino acid interchanges require only one nucleotide base change at the DNA level. To date, comparision of the molecules has shown two regions (positions 61-82 and 87-105) of less than 50% homology and in which many of the amino acids require multiple base changes for interconversion. These regions may be involved in alloantigenic specificity.

Comparison of the $H\text{-}2K^b$ amino acid sequence to the primary structure of other proteins reveals that an internal region of the molecule encompassing the second disulfide is homologous to the constant region domains of immunoglobulin heavy and light chains as well as β_2-microglobulin. This indicates that all these molecules of the immune system evolve from the same ancestral gene.

Studies on the $H\text{-}2D^b$ (H-2.2) and $H\text{-}2D^d$ (H-2.4) molecules have progressed to the point where the CNBr peptides of the H-2 molecules have been isolated and aligned by homology to $H\text{-}2K^b$. NH_2-terminal sequence analyses of each of these peptides has allowed the assignment of residues

which account for approximately 40% of the total sequence. The homology of these molecules to the H-2K^b molecule is approximately 85% for the positions available for comparision.

Similar studies on the H-2K^d and H-2L^d molecules have been initiated. Because of their proximity in the genome, it has been difficult to differentiate the H-2D^d and H-2L^d molecules. NH_2-terminal sequence analysis of the H-2L^d indicated that this molecule can be distinguished from H-2D^d by its lack of a valine and phenylalanine at positions 9 and 17, respectively, and by the substitution of an isoleucine for a methionine at position 23.

The determination of the amino acid sequence of murine β_2-microglobulin is 95% complete and, as expected, it's primary structure is highly homologous (~80%) to that of β_2M from other species.

A major goal of our structural studies on the H-2 glycoproteins is to locate positions of structural changes in the H-2 molecules isolated from the series of H-2K and H-2D utants of the b and d haplotypes. Strains of each K and D mutant series show strong histogenic reactivity in vivo and MLR and CML in vitro with the parents. In five of the H-2K^b mutants that have been examined, there appear to be only one or two amino acid interchanges between the H-2K molecules, produced by the parent and mutant strain. Further studies on the precise nature of the amino acid interchanges in these and other H-2K molecules of this mutant series will serve to localize the region of the molecule in which polymorphism can serve as a specific and effective signal for recognition by the immune system.

An added impetus to the study of these molecules is given by the relationships in humans between the homologous loci (HLA A and B) and predisposition to certain diseases. Furthermore, molecules coded for by the K and D loci of the major histocompatibility complex appear to play a major role in the stimulation and specificity of T-lymphocyte response to virally induced and other cell surface antigens.

Publications

Coligan, J.E., Kindt, T.J., Ewenstein, B.M., Uehara, H., Nisizawa, T., and Nathenson, S.G. Primary structure of murine MHC alloantigens: II. Amino acid sequence studies of the cyanogen bromide fragments of the H-2K^b glycoprotein. Proc. Natl. Acad. of Sciences, USA 75: 3390-3394, 1978.

Coligan, J.E., Kindt, T.J., Ewenstein, B.M., Uehara, H., Martinko, J.M., and Nathenson, S.G. Further analysis of the murine H-2K^b glycoprotein using radiochemical methodology. Mol. Immunol. 16: 3-8, 1979.

Kindt, T.J., Coligan, J.E., Kimball, E.S, Ewenstein, B., Uehara, H., Martinko, J., and Nathenson, S.G. Use of radiochemical techniques for primary structural analysis of mouse and rabbit histocompatibility antigens. Proceedings of the Josiah Macy Foundation, in press, 1979.

Nathenson, S.G., Ewenstein, B.M., Uehara, H., Martinko, J.M., Coligan, J.E., and Kindt, T.J.: Recent Studies on the Structure of the H-2K^{b} Glycoprotein and on the H-2K MHC Mutants. In Ferrone, S. and Resfeld, R.A. (Eds.): Current Trends in Histocompatibility. Plenum Press, 1979, in press.

Uehara, H., Ewenstein, B., Martinko, J.M., Nathenson, S.G., Coligan, J.E., and Kindt, T.J. Primary structure of murine MHC alloantigens: amino acid sequence of the amino terminal 173 residues of the K^{b} glycoprotein. Biochemistry, in press, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00170-02 LIG

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Structural Studies on Precursors to Immunoglobulin Molecules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Thomas J. Kindt	Chief, LIG	LIG	NIAID
Others:	Dr. Frederick T. Gates	Staff Fellow	LIG	NIAID

COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Immunogenetics

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.5	0.3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The in vitro translation of the mRNA for some proteins yields products which are larger than those found in vivo. The amino termini of these precursor proteins are extended by up to thirty amino acids when compared to their mature counterparts. Precursor sequences have been reported for murine (plasmacytoma) immunoglobulin light and heavy chains, with the conclusion that these sequences represent extensions of the variable region genes. In order to better understand the multi-gene nature of immunoglobulin genes, the purpose of this work is to sequence other mouse immunoglobulin precursor molecules for comparison and to establish the nature of such sequences in the rabbit. The latter goal requires the construction of hybrid cell lines capable of secreting rabbit immunoglobulin chains as sources for the isolation of rabbit immunoglobulin mRNA. This mRNA must then be isolated and translated in vitro, and the translation products must be analyzed using radiosequence techniques.

21-17

Structural Studies on Precursors to Immunoglobulin Molecules

Peptides of as many as thirty amino acids may be present at the amino terminus of a protein when it is synthesized and these peptides may then be cleaved from the rotein, presumably durng the secretion process. These precursor peptides, which are hydrophobic, have been identified for hormones, digestive track enzymes, and serum proteins. It is postulated that these peptides direct the passage of the nascent polypeptide chain through the membrane of the endoplasmic reticulum.

Precursor peptides for several BALB/c mouse immunoglobulin light chains and a single heavy chain have been previously reported. The amino acid sequence data have indicated that light chan precursors appear to be somewhat homologous within and between light chain subgroups, while much less homology exists between precursor peptides of Kappa and lambda light chains. In collaboration with A. Williamson and H. Singer (University of Glasgow) we have determined a partial amino acid sequence for the precursor peptides of a gamma heavy chain and a Kappa light chain from a C3H mouse myeloma. These sequences are not homologous to those reported for BALB/c mice, indicating a broader diversity of precursor peptide sequence than was previously assumed.

In order to study the structure of precursor peptides of immunoglobulins from another species, we are constructing mouse-rabbit hybrid cell lines (hybridomas) which secrete rabbit immunoglobulin chans. With these cell lines as sources of mRNA for in vitro protein synthesis, we are immunoprecipitating the subsequent rabbit immunoglobulin products prior to radiosequence analysis. The multiplicity of kappa constant region genes in the rabbit allows a study of gene rearrangement and regulation of gene expression which is not possible in the mouse system. This analysis will help to define the structure-function relationship of immunoglobulin precursor peptides and the genes which encode them.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00171-02 LIG

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Genetic Studies on Rabbit Immunoglobulins and Other Serum Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Others:	Dr. Thomas J. Kindt	Chief, LIG	LIG	NIAID
	Dr. Martin Yarmush	Chemist	LIG	NIAID
	Mr. Mark Simpson	Veterinary-Costep	LIG	NIAID

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Dr. John Coe, Rocky Mountain Laboratory, NIAID

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.35	0.5	0.85

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The serologic markers of rabbit immunoglobulins-allotypes and idiotypes, are under study because they can provide information concerning the number and arrangement of genes encoding the immunoglobulins. A major effort has been made to characterize allotype $b4^{V}$, a new allele of the group b allotypes of the C_{K} region. This allotype, first discovered in this laboratory during amino acid sequence studies, has now been shown by genetic analysis to be an allele of the other group b allotypes and has been characterized serologically. The utility of chain specific idiotypes for genetic studies in the rabbit has been confirmed, and the initial study of an L chain specific idiotype has been extended with an H chain-specific idiotype. It was possible to show linkage of this idiotype to V_{H} and C_{H} allotypes within a single allogroup, and to demonstrate latent expression of an allogroup. Attempts to improve breeding efficiency of inbred rabbits are underway at present. Idiotypy studies will be extended to these animals when sufficient numbers are available.

PHS-6040
(Rev. 10-76)

Genetic Studies on Rabbit Immunoglobulins and Other Serum Proteins

The various serologic markers of rabbit immunoglobulins have been valuable probes for the study of genetic interactions involved in the humoral immune response. The rabbit has a uniquely diverse repertoire of allotypes (intra-species antigenic variants), encompassing most regions of the antibody molecule. The structural correlates for some allotypic markers are simple, while for others they are either complex or unknown. Studies within this project are designed to expand the allotypic repertoire, to increase its structural definition, and to use these serologic markers to examine questions about the number of genes involved in immunoglobulin synthesis and the mechanisms controlling interactions among these genes. The studies based on allotypes have been complemented and extended by use of idiotypes as markers for the antibody combining site. The obvious complexity of idiotypes as genetic markers has been lessened somewhat by increasing the specificity of idiotypic antisera, either by fractionation based on antigen competition or by limiting idiotypic recognition to determinants on one of the antibody chains.

Serologic characterization is now complete for a new C_k allotype, $b4^v$, which has been under study for two years. While conventional antiallotypic reagents do not distinguish b4 from $b4^v$, three methods of serologic distinction have been found. First, as reported previously, one anti-b4 serum, upon adsorption on a column containing bound cottontail rabbit IgG, yielded an antibody fraction which reacted well with b4 IgG but poorly with $b4^v$ IgG. An inhibition of binding radioimmunoassay could then be used to differentiate $b4^v$ from b4. Second, in collaborative studies with Dr. John Coe, several bull-frog antisera raised against $b4^v$ IgG or crosslinked $b4^v$ L chains reacted specifically with $b4^v$ IgG after adsorption with b4 IgG. The reaction could be detected either by radiobinding or precipitin assays. Although only low levels of activity were found, the result established that $b4^v$ IgG does possess antigenic determinants not found on b4 IgG. Third, collaborative studies with Dr. Jan Naessens have proven that $b4^v$ and an allotpe described in Belgium, b4.2, are serologically and structurally identical. This finding has made the serologic detection of $b4^v$ in serum much easier because an antiserum exists (anti-MS7) which reacts only with IgM containing L chains of allotype b4.2 (or $b4^v$). The reaction can be seen readily by precipitin reaction. Another serum of the MS series, anti-MS3, reacts in a similarly specific way with IgM containing b4.1 (b4) L chains. Structural identity of $b4^v$ with b4.2 was shown by sequence analysis of succinylated, acid cleaved L chains.

Further genetic analysis of the rabbit immune response will be difficult without rabbit of more defined genetic composition. To take advantage of available in vitro techniques for dissecting the cellular components of the unimmune response fully inbred rabbits would be particularly valuable. Some attempts to incorporate existing inbred rabbits into studies in ths laboratory have been made in the past year. The B/J strain has been examined to determine optimal parameters for

immunzation with antigens inducing monoclonal responses. Idiotypic analysis will be done to examine idiotypic diversity in an inbred population. Large scale studies will require numbers of B/J rabbits not available with current breeding techniques. Methods for enhancing fertility and reproductive rate are being examined in cooperation with the Veterinary Resources Branch and Dr. Ben Wolf.

Limited structural studies are underway in collaboration with Dr. John Coe on a protein isolated by Dr. Coe from the serum of female hamsters. The protein is present only in trace amounts in nude hamsters. Amino acid composition and sequence analysis are being used to determine whether the female protein exhibits structural homology to biological active proteins of several types already sequenced in other species.

Publication

Sogn, J.A. and Kindt, T.J.. Genetic characterization of a new allele of the rabbit group b C_K allotypes. Immunogenetics 7: 141-147, 1978.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00172-02 LIG

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Carbohydrate and Glycoprotein Antigens of Microbial Cell Walls

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Dr. John Coligan Research Microbiologist LIG NIAID

COOPERATING UNITS (if any)
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Previous compositional and immunological studies have defined the chemical and antigenic structure of the group-specific carbohydrates for Groups A, A variant and C streptococci. These polysaccharides were shown to have a common core structure. Work is in progress to determine the structure of the group specific carbohydrate of the Group B streptococcus in order to chemically define its antigenic determinants and to determine its chemical relationship to the other streptococcal group specific carbohydrates.

PHS-6040
(Rev. 10-76)

Publications

Coligan, J.E. and Slayter, H. Physical, chemical and immunological characterization of saline-extracted, concanavalin A purified carcinoembryonic antigen. Mol. Immunol. 16: 129-135, 1979.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00173-02 LIG

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Nonallelic Expression of Genes Encoding Rabbit Immunoglobulins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Dr. John A. Sogn	Chemist	LIG NIAID
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	1.3	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Rabbit immunoglobulin allotypes are antigenic determinants thought to be inherited as autosomal co-dominant alleles. This notion has been challenged by observations of low concentrations of allotypes not detected by qualitative tests or predicted by parental genotypes. In the past year, L chains with "latent" allotypes were isolated from the sera of pedigreed rabbits and shown by amino acid sequence analysis to be indistinguishable from the normal allotypes. Current research focus on hybridoma cells maintained in culture that synthesized rabbit immunoglobulin RNA will be isolated from these cell lines and cDNA probes will be prepared to carry out molecular biological studies in order to determine the basis for inheritance of latent allotypes. Other studies in this area have included clearance of normal versus latent allotypes in animals using doubly labelled material and studies on the linked expression of V_H markers on immunoglobulins expressing latent allotypes.

PHS-6040
(Rev. 10-76)

Nonallelic Expression of Genes Encoding Rabbit Immunoglobulins

In the past year, studies on latent allotypes of rabbit immunoglobulins have included complete serologic characterization of allotypic markers of both the constant and variable regions of the rabbit molecules. It has been shown by structural studies that the molecules expressing latent allotypes have markers that are indistinguishable from those of the allotypes normally expressed. Latent Group b markers of the constant region of the rabbit antibody light chain were previously detected in sera, in IgG preparatins, and on isolated L chains from rabbits bred for homozygosity at the b locus. Serologic analysis of sera from an extended family of homozygous b4 rabbits revealed the presence of latent b allotypes in 5 of 37 sera tested. Latent b5 and b9 markers were identified. None of the sera tested contained latent b6. In two instances, the level of latent b9 allotype was sufficiently high to permit isolation and detailed characterization of the immunoglobulin population bearing this latent allotype. These studies which are completely described in another section indicated that the nominal and latent allotype light chains were identical in sequence.

Studies concerning the linked expression of C_H and V_H allotype genes have involved isolation of molecules on the basis of a latent group a allotype. This is carried out by use of immunoadsorbent chromatography employing specific antibodies directed against the allotype specificities. The isolated molecules were then typed for their group d markers (d11, d12) by serologic and by chemical means. Approximately half of the molecules isolated were shown to carry latent group d markers in addition to the latent group a allotype. However, in no case was found a molecule with combinations of group a and group d markers that are not normally expressed in the rabbit populations studied. This finding indicates that the mechanism for synthesis of latent allotypes are similar to those for nominal allotypes and do not represent products of events such as misspairing of multiple genes involved in H chain synthesis.

A promising new tool for the study of latent allotypes has been introduced by the use of interspecies (mouse-rabbit) hybridoma cells. Cells have been prepared that will secrete rabbit heavy or light chains in culture and in ascites fluid of nude mice. These cells are being prepared in bulk culture and RNA encoding the rabbit immunoglobulin chains will be isolated and used to prepare cDNA which can be used as a probe to study the occurrence of latent allotype genes in various rabbits.

IgG clearance rates were measured by intravenous injection of rabbits with differentially labelled (^{125}I and ^{131}I) allotype matched and nonmatched IgG samples. In no instance was the allotype matched IgG cleared faster than nonmatched although the converse was true in several instances. These data suggest that there is an *in vivo* recognition of allotypes which is a possible regulatory mechanism. Many questions remain to be answered concerning mechanisms for the regulation of synthesis and the selection of genes involved in the preparation of normal and latent allotypes.

Publications

Yarmush, M.L. and Kindt, T.J. Isolation and characterization of IgG molecules expressing latent group b allotypes from pedigreed b^4b^4 rabbits. J. Exp. Med. 148: 522-533, 1978.

Mudgett-Hunter, M., Yarmush, M.L., Fraser, B.A., and Kindt, T.J. Rabbit latent group a allotypes: Characterization and relationship to nominal group a allotypic specificities. J. Immunology 121: 1132-1138, 1978.

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Wolf, B., Urban, R., Miller, A.B., Kimball, E.S., Mudgett, M., Catty, D., and Danemann, J.: Nonallelic inheritance of V_H region a group allotypes. Cell surface and serum studies in double and triple expressing rabbits. J. Immunol., in press, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00180-01 LIG

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Monoclonal Antibodies Directed Against Cell Surface Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. John A. Sogn	Chemist	LIG	NIAID
Others:	Dr. Thomas J. Kindt	Chief, LIG	LIG	NIAID
	Dr. Edward S. Kimball	Staff Fellow	LIG	NIAID
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COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Immunogenetics

SECTION
Immunogenetics Research Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	1.2	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Preliminary studies are under way on antibodies to cell surface proteins produced by hybridoma techniques. The initial targets for this investigation is cell line RL-5, a rabbit T-cell tumor. The cell line has been characterized by conventional techniques and several mouse-mouse hybridomas with activity against this cell have been produced, cloned and propagated in vivo and in vitro. Biochemical characterization of the molecular specificity of these antibodies is under investigation.

PHS-6040
(Rev. 10-76)

The ability to produce monoclonal hybridoma antibodies, in unlimited amounts, directed against any antigenic determinant of interest, no matter how impure, has revolutionized the study of the biologically important proteins of the cell surface. These proteins are difficult or impossible to purify by conventional techniques because they are present in vanishingly small amounts and because their isolation is complicated by the hydrophobic nature of these integral membrane constituents. Characterization of such proteins has been feasible only by indirect methods using genetically defined organisms, where specific antibodies may be produced by cross-immunization of antimals differing at a limited number of genetic loci. Structural studies in outbred species such as rabbit and man have, therefore, been especially limited. Hybridoma technology removes these restrictions. The research interests of several groups in the Laboratory of Immunogenetics currently involve molecules of immunologic importance found on cell surfaces. The goal of this project is to raise hybridoma antisera against these molecules so that structural studies can proceed rapidly.

The principal current interest is the rabbit cell line RL-5. This virus-induced lymphoma has been characterized as a member of the T-cell lineage on the basis of reactivity with two specific anti-rabbit thymocyte sera, the absence of cytoplasmic and surface immunoglobulin, and the absence of receptors for Fc or C3. Mice have been immunized with RL-5 and spleen cells from the immunized mice have been fused with appropriate myeloma cells to obtain hybrid antibodies against RL-5. Antibody activity is measured in an ELISA assay. The membrane components against which the antibodies react are currently being identified by immunoprecipitation and electrophoretic techniques.

Publication

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-A1-00191-01 LIG

PERIOD COVERED
New - October 1, 1979

TITLE OF PROJECT (80 characters or less)
Immunoregulation of T Lymphocytes - The Role of Anti-idiotype and Immune Complexes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Kenneth W. Sell	Head, Immunobiology Section	LIG, NIAID
Other: Tom Folks	Staff Fellow	LIG, NIAID

COOPERATING UNITS (if any)

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3- 1/2	1- 1/2	2

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Investigation of mechanisms by which immunoregulatory signals are transmitted to T-lymphocytes to cause activation or suppression of their activity. Project attempts to examine both antigen and allogeneic receptors on T-lymphocytes and the means by which they may be activated, blocked or stimulated to active suppression of T-cell functions In particular, the role of immune complexes and the related role of anti-idiotype antibodies in the regulatory process will be examined. The interaction between antigen, antibody, complement, anti-idiotype immunoconglutinin and rheumatoid factor will be examined in the make-up of immune complexes to determine the role of each, individually or acting in concert, to produce effects on T-cell activation or effector function. In addition, attempts will be made to identify immune complexes in disease states such as juvenile diabetes and hepatitis to determine what role they may play in vivo in the suppression of cell-mediated immunity in these diseases which are related to latent or persistent viral infections.

(21-29)

Project Description

Objectives

The specific suppression or inactivation of those clones of antigen-reactive cells directed against foreign antigens or donor histocompatibility antigens has been the object of intensive research. A recent finding has been the disclosure that F_1 hybrids between two inbred strains of mice will synthesize anti-allo-antibodies if injected with either parental lymphoid cells or in allo-antiserum produced in the same parental strain against the allo-antigens of the other parental strain. These observations have led to the concept that the recognition of allo-antigen by the T-lymphocyte is the primary step towards allograft rejection and initiation of antigen responsiveness. The exact nature of the T-lymphocyte recognition structure is still an unresolved question. Binz and Wigzell have obtained evidence that suggest that the recognition structure is similar,if not identical, to the combining site of allo-antibody (idio-type). Isolated T-cell receptors have been shown to have a single chain structure with a molecular weight of 70,000 daltons. It has been suggested that both T and B cells employ a common pool of heavy chain V genes in synthesis of the antigen binding site on the respective antigen receptors. The use of the term "idiotype" for the recognition structure on T-cells is based on the evidence for shared idiotypic determinance on both T and B cells. Both normal T-cells as well as T-lymphoblasts which have been specifically activated by antigen or the mixed lymphocyte reaction both express idiotypic determinants against the relevant histocompatibility or foreign antigen. Although these idiotype-positive cells specific for a given set of major histocompatibility complex antigens are found with very low frequency, less than 3 percent, in the normal resting population of peripheral blood lymphocytes, they are greatly enriched during the in vitro proliferation in an MLC response against the relative MHC.

Auto and anti-idiotype (anti-receptor) antibodies can be produced by immunization with lymphoblasts. These can deplete the responder T-lymphocyte population apparently through cytotoxic killing in the presence of complement. They are not capable of blocking MLC reactions in the absence of complement as they apparently compete ineffectively for binding with the receptor site on T-cells responsible for the MLC reaction.

Inhibitors of T-cell mediated immunity have been identified in certain clinical diseases. In subacute sclerosing panencephalitis (SSPE) and in CMV virus disease, immune complexes have been identified which apparently are capable of specifically inhibiting T-cell responses to the virus and specifically blocking effector functions such as the release of macrophage inhibition factor. We hypothesize that these immune complexes may exert their immunosuppressive activity on T-lymphocytes because of the presence of anti-idiotypes. It is possible that the immune complex serves as a focusing mechanism or perhaps a stabilizing mechanism allowing the more effective interaction of anti-idio-type with T-cell receptors, thus providing for blocking or initiation of inactivation events in the T lymphocyte.

We, therefore, propose to study the role of anti-idiotype and immune complexes as they interact with receptors on T-lymphocytes in order to elucidate the

mechanisms of immunoregulation of cell-mediated immune events.

Methods Employed:

1. Attempts will be made to construct model immune complexes in order to evaluate their interaction with T-cell receptors and subsequent T-cell activity. Specific antibody and anti-idiotype as well as immunoconglutinin and rheumatoid factor will be prepared using hybridomas. Balb/C mice will be immunized with specific carrier proteins such as BSA, OVA, BGG, KLH, etc., and their spleen cells fused with appropriate mouse myeloma cells in order to establish specific clones of antibody producing cells. The Elisa technique will be utilized to assay for the presence of specific antibodies. It is anticipated that spleens of immunized animals will provide cells producing, not only specific antibody against the relevant antigen but also cells expressing anti-idiotype against antibody. In addition, autoantibodies against C-3 (immunoconclutinin) and an auto-antibody against IgG (rheumatoid factor) will be anticipated to occur in the immunized mice. Each of these antibodies then can be used along with purified complement (C3) to construct immune complexes which can be tested in cell-mediated immune tests both for recognition (transformation) or for effector function (MIF assay) or killer cell assays.

2. Lymphocytes and serum will be collected from chimpanzees which have been infected with hepatitis virus. The lymphocytes will be assayed for cell-mediated immune activity including transformation and MIF production. Serum will be assayed for the presence of inhibitors of cell-mediated immunity. If inhibitors are discovered, specificity will be determined and nature of the inhibitor will be examined with particular emphasis on techniques to identify immune complexes. Assays will be made regularly during the infective stage of the disease in the chimpanzee to determine the sequential appearance of cell-mediated immunity and inhibition.

3. Patients with juvenile diabetes as well as control patients will be tested for the presence of humoral and cell-mediated immunity against viruses which have been suspected in the etiology of diabetes. In particular, coxsachie B4 will be evaluated and cell-mediated immunity (transformation and MIF) will be studied and compared with a control group of sex and age matched normal children. Serum of diabetic patients will be examined for inhibitors of cell-mediated immunity against candidate viruses with immunochemical studies to determine the presence of immune complexes.

LABORATORY OF IMMUNOLOGY
1979 Annual Report
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PHS-NIH
Summary Statement
Office of the Chief
Laboratory of Immunology
October 1, 1978 through September 30, 1979

Recombination between Genes for μ and δ Immunoglobulin Heavy Chains

The genes controlling the constant regions of heavy (H) immunoglobulin (Ig) chains of various classes exist as a genetic linkage group termed the IgC_H complex in the mouse. The genes specifying IgA and the various IgG H chains are very tightly linked. Indeed, in the examination of several thousand individual mice and large numbers of humans, no instance of a genetic recombination separating these genes had been observed. It is known that genes for the μ and δ H chains are also found in the IgC_H complex but the degree of linkage has not been extensively studied because allotypic markers for the μ and δ genes have only recently been described. In the course of experiments aimed at studying the linkage of Lyb7 and IgD, scientists in the Laboratory of Immunology and at the Naval Medical Research Institute, have observed two instances of recombination between the IgG_{2a} and IgD genes. The initial observation was that two progeny (numbers 72 and 74) of a cross between (C57BL/6 x DBA/2) and C57BL/6 possessed the "DBA/2" allele of IgD [IgD^a] but lacked the "DBA/2" allele of IgG_{2a} [IgG_{2a}^c]. This phenotype was transmitted to progeny on subsequent crossings of individuals 72 and 74 to C57BL/6 to create families 72 and 74. Proof that the failure of these mice to express IgG_{2a}^c was due to its replacement by IgG_{2a}^b [the allele possessed by C57BL/6] was obtained by crossing members of families 72 and 74 to A mice and demonstrating that progeny that inherited IgD^a, which is distinguishable from the IgD^e of A, also inherited IgG_{2a}^b. It has also been possible to show that in both recombinants the genes for IgG_1, IgA, IgM and prealbumin segregated with the IgG_{2a} gene. These recombinant families represent the first documented examples of intra-IgC_H genetic recombination. Studies of segregation of IgV_H markers in these mice, which are now in progress, will allow construction of more detailed genetic maps of this genetic region, which is of key importance in the specification of antibody molecules. In turn, this will prove of considerable utility in understanding the genetic basis of the immune response (Subbarao, Lieberman and Paul, LI/NIAID; Ahmed and Scher, Naval Medical Research Institute).

Genetic Regulation of the Anti-fructosan Antibody Response

Bacterial levan (BL) is a β (2→6) linked polyfructosan with β(2→1) branch points. Anti-BL antibodies produced in BALB/c mice consist of two families of molecules. One of these bind BL but not inulin, a β (2→1) polyfructosan, and thus appear to be specific for β (2→6) fructosans. The other set of antibodies bind inulin and BL, are principally specific for β (2→1) linkages and express one or more of a family of cross reactive idiotypes (IdX) found on inulin-binding myeloma proteins of BALB/c mice. It has previously been shown that the capacity to produce both anti-inulin antibody and IdX-bearing molecules in response to immunization with BL requires the presence of genes linked to the IgC_H gene complex. During the past year, Laboratory of Immunology scientists have demonstrated that non-IgC_H genes exert major effects on the

amount and clonal diversity of the anti-inulin antibodies produced in response to BL immunization. Thus, BALB/c mice immunized with BL produce inulin-binding antibodies which, when analyzed by isoelectric focusing (IEF), consist of characteristic "triplet" of bands. C57BL/6 mice, or C.B20, which are BALB/c congenic mice expressing $\underline{IgC_H}$ genes of C57BL/6, make no anti-inulin antibodies after BL immunization. Nonetheless, F_1 hybrids between C57BL/6 and BALB/c make a more vigorous and more diverse anti-inulin response to BL, as judged by IEF, than do BALB/c. The genetic influence contributed by the C57BL/6 depends on genes outside the Ig H chain genetic region since B.C8 mice, which are congenic to C57BL put possess BALB/c $\underline{IgC_H}$ genes, make a response similar to the F_1 in its amount and diversity. Through the analysis of amount and diversity of the anti-inulin response to BL of recombinant inbred lines between C57BL/6 and BALB/c, it has been tentatively concluded that two $\underline{non\text{-}IgC_H}$ genes regulate the clonal diversity and amount of the anti-inulin response. These studies should shed considerable light on the genetic factors which regulate immune responses to simple antigens, particularly those which are excellent models for polysaccharide antigens used in bacterial vaccines (Stein, Bona, Lieberman and Paul, LI/NIAID).

A Specialized Subset of B Lymphocytes is Stimulated By Anti-Immunoglobulin Antibodies

One of the most direct and potentially informative approaches to the study of the requirements for B lymphocyte activation is the use of antibodies to B lymphocyte membrane receptors to stimulate these cells. Laboratory of Immunology scientists have established that specifically purified antibodies to μ H chains and to κ L chains cause marked stimulation of proliferative responses by B lymphocytes. The response is independent of any requirement for T lymphocytes or macrophages. However, anti-Ig antibodies do not stimulate the synthesis of Ig by B lymphocytes; indeed, these antibodies are powerful inhibitors of stimulation of Ig synthesis by both antigens and polyclonal B lymphocyte activators. During the past year, scientists in the Laboratory of Immunology and the Naval Medical Research Institute have shown that the capacity of lymphocytes to respond to anti-μ and anti-κ is a property of a specialized subset of B lymphocytes which has a characteristic density of membrane (m) Ig and expression of differentiation antigens. To show this, B lymphocytes were incubated with fluorescein (Fl) conjugated anti-Ig, anti-μ or anti-δ reagents and separated into populations with characteristic densities of mIg using the fluorescence activated cell sorter (FACS). Cells with a high density of total Ig, a relatively low density of IgM, and a moderate to high density of IgD were most responsive to anti-Ig. By contrast, cells with a low density of total Ig, a high density of IgM, and a low density of IgD were poorly responsive. The results also suggest that the bulk of cells responsive to anti-Ig are $Lyb5^+$. These results establish that responsiveness even to simple stimulants such as anti-μ is a property of a discrete B lymphocyte subpopulation. Further progress in delineating the biochemical events in B lymphocyte activation by specific stimuli will require techniques to purify and/or clone specific populations of B lymphocytes. Sieckmann and Paul, LI; Scher, Naval Medical Research Institute).

Failure to Detect V_H Framework Determinants on T Lymphocytes

The delineation of the chemical nature of the antigen-binding receptors of T lymphocytes has been one of the most challenging and critical problems of modern immunology. There is an increasing body of functional and genetic data which indicates that some idiotypic determinants, presumably markers of hypervariable and J segments of immunoglobulin H chains, are expressed on T lymphocyte receptors as well as on antibodies and B lymphocyte receptors. These findings provide strong evidence that structural genes in the IgV_H genetic region code for at least part of the T lymphocyte receptor. Laboratory of Immunology scientists have sought to determine whether antigenic determinants associated with framework areas of Ig H chain variable (V_H) regions were also expressed on T lymphocytes. In order to examine this, anti V_H framework antibodies against allotypic and species-specific determinants have been prepared. The former are the well known antibodies to a locus allotypic determinants; the latter are antisera to rabbit V_H determinants prepared in goats. Such reagents have been fluoresceinated and their ability to stain B and T lymphocytes determined by direct examination and by use of the fluorescence activated cell sorter (FACS). Thus far, no evidence for T lymphocytes expressing V_H determinants has yet been obtained. Since T lymphocytes have been shown to lack classical light (L) chains, efforts have been made to chemically identify V_H bearing molecules which lack L chains. Again, no such molecules have yet been found. This data is consistent with the failure of V_H framework determinants to be expressed on T lymphocytes at all, even on T lymphocytes which appear to express idiotypic determinants. It is also consistent with the possibilities that only a very small fraction of T cells possess V_H-bearing receptors or that each T cell expresses very small amounts of such receptors. These studies are being pursued both with an effort to increase their sensitivity and to obtain data on the effects of anti-V_H sera on T lymphocyte functions. They promise to offer important insights into the nature of antigen-recognition by T lymphocytes (Wilder and Mage, LI/NIAID; Scher, Naval Medical Research Inst.).

Phospholipid Methylation is an Early Event in T Lymphocyte Activation

T lymphocytes can be stimulated to proliferate by exposure to a wide variety of agents of which the plant lectin concanavalin A (Con A) is a prototype. It has long been known that many intracellular events are associated with stimulation of DNA synthesis by Con A but no clear understanding of the critical initial events which lead to the proliferative response has yet been developed. Recently, scientists in the Laboratory of Immunology and in the National Institute of Mental Health have shown very rapid changes in membrane phospholipid methylation following exposure of T lymphocytes to Con A. Thus, there is a maximum incorporation of ^3H-methyl groups into membrane phospholipids within 10 minutes after the addition of Con A followed by a somewhat slower calcium dependent degradation so that normal levels of methylation are found at 40 minutes. Increases in phospholipid methylation are closely correlated with subsequent proliferative events. Thus, the dose-response curve for increased phospholipid methylation and for stimulation of DNA synthesis are similar; only cell types which proliferate in response to Con A exhibit increased methylation; and, only

those lectins which are mitogenic induced methylation. Furthermore, inhibition of methylation by addition of a methylation-inhibitor prevents subsequent DNA synthesis but only if the inhibitor is added before the increase in methylation caused by Con A. These results suggest that membrane transmethylases are activated by binding of Con A to the cell, that this leads to an increase in several methylated phospholipids including monomethylethanolomine. Such an increase may lead to decreased membrane viscosity and the entry of calcium into the cell. Calcium is apparently associated with the accelerated degradation of methylated phosphiolipids and release of a series of compounds, such as thromboxane, prostaglandins, and lysolecithin which may be of considerable importance in subsequent events of lymphocyte activation. These early biochemical events appear to be excellent candidates to be critical steps in the cellular processes which determine the capacity of physiologic stimulants, such as antigens, to activate lymphocytes (Toyoshima and Waxdal, LI/NIAID; Hirata and Axelrod, NIMH).

Antigen Presentation by Macrophages: both Antigen and Ia Gene Products must be Expressed on the Surface of the Antigen-presenting Cell

The activation of proliferative responses and of lymphokine production by specific T lymphocytes depends on presentation of antigen by a specialized macrophage-like cell. Antigen-recognition by T lymphocytes has been shown by Laboratory of Immunology and Laboratory of Clinical Investigation scientists to involve recogntion of both I-region associated (Ia) major histocompatibility complex antigens and the conventional antigen to which the donor of the T lymphocytes had been primed. The role of Ia antigen recognition in this process has been strongly supported by the finding that anti-Ia antisera are potent inhibitors of T cell activation. Paradoxically, antisera to the "conventional" antigen have been uniformly ineffective in the blocking of T lymphocyte activation by antigen-pulsed macrophages. Although this failure of antibody to cause inhibition has certain physiologic advantages, it also raises the possibility that the stimulatory antigen may not be on the surface of the antigen-presenting cell. To further study this critical point, Laboratory of Immunology scientists have developed a T lymphocyte activation system in which the antigen-presenting cell is covalently derivatized with trinitrophenyl (TNP) groups and responding T cells are primed to TNP-macrophages in vivo or in vitro. The antigen-presenting retains its capacity to stimulate responses by T lymphocytes even if it is incubated for 24 hours after derivatization before addition to T cells. Despite the capacity of these "aged" TNP-macrophages to stimulate a TNP-specific response, anti-TNP antibody has no inhibitory, effect on the activation process. However, if the macrophage is subject to a second round of derivatization with either TNP or DNP, just prior to being added to the T cells, its stimulatory activity is now sensitive to inhibition by anti-TNP antibody. This is particularly striking for the case in which DNP derivatization is used because DNP-derivatized macrophages do not stimulate responses by T cells primed to TNP-macrophages, although, anti-TNP antibody does bind DNP. This result provides strong evidence that the stimulatory antigen is on the surface of the antigen-presenting cell. It appears the antigen is present at a density too low

to be cross-linked and cleared from the surface by anti-TNP antibody. The second round of derivatization increases the antigen density and thus leads to the result that the cell is now susceptible to blockade. However, these results still lead to the conclusion that the antigen on the macrophage, surface can stimulate T cell responses even in the presence of saturating amounts of antibody. These results have considerable significance in the understanding of the regulation of T lymphocyte immunity by antibody and in the elucidation of the molecular events in antigen recognition. (Thomas and Shevach; LI/NIAID).

Cellular Expression of Responder Phenotype in Complementing Immune Response Gene Systems

Specific immune response (Ir) genes of the major histocompatibility complex control the ability of T lymphocytes to be activated by specific antigens. In certain systems, such as responses to poly(Glu,Lys,Phe) [GLΦ] and to pigeon cytochrome C, responsiveness depends upon the possession of responder alleles of Ir genes in both the I-A and I-E/C subregions of the mouse MHC. It had previously been shown in these and related systems that antigen-specific T lymphocyte activation by antigen-presenting cells (APC) required that the donor of the APC be a responder to the antigen under study. However, whether the responding T cell had to be derived from a responder donor was uncertain. Laboratory of Immunology scientists have studied this problem through the creation of bone marrow chimeras. Thus, F_1 hybrids prepared by crossing complementing non-responder parents (i.e. $Ir\text{-}GL\Phi\alpha^{+}\beta^{-}$ and $Ir\text{-}GL\Phi\alpha^{-}\beta^{+}$ mice) are capable of responding to GLΦ. If these mice are lethally irradiated and reconstituted with bone marrow from both parents, the resulting mice are unresponsive to immunization with GLΦ, indicated that some cell must possess both complementing Ir genes in order for a response to ensue. However, if T lymphocytes are transferred from such a reconstituted mouse to a lethally irradiated F_1 recepient together with a source of F_1 APC and immunized immediately with GLΦ, they respond to GLΦ. This indicates that the inability of non-responder T lymphocytes to respond is not a genotypic character of the T cells, but depends, at least in part, upon cells in their sensitizing microenvironment. On the other hand, when bone marrow cells from F_1 (responder) donors were used to reconstitute lethally irradiated mice of either of the non-responder parental types or of the responder F_1 type, only the $F_1 \rightarrow F_1$ chimera could subsequently respond to antigen. This indicates that the developmental, as well as the sensitizing, microenvironment is critical for the acquisition of the responder phenotype by the T lymphocytes. These results provide further insight into the nature of the reagulation of immune responses by MHC Ir genes and should allow a better understanding of the linkage of disease susceptibility and resistance to MHC genes. (Longo and Schwartz, LI/NIAID).

Three Cells Participate in the In Vitro T Lymphocyte Proliferative Response to Antigen

It is now well recognized that antigen-specific activation of T lymphocytes for proliferative, lymphokine producing, or helper responses can be

effeciently achieved by the presentation of antigen to the T cells in association with an Ia^+ macrophage-like cell. The analysis of this antigen-presentation system has led to the demonstration that T lymphocytes recognize both antigen and I-region gene products expressed on the surface of the antigen-presenting cell. Nonetheless, this analysis only demonstrates that cellular interactions are important in the artificial condition of addition of antigen-pulsed cells to a primed T lymphocyte population. It does not give a picture of the relative importance of such interactions in the normal in vitro response of unseparated lymphoid cells.

In order to examine cellular interactions in unseparated cell populations, Laboratory of Immunology scientists have studied the relation between numbers of cells cultured and response to added antigens. In an ideal situation, the diminution of response upon diluting a cell population by a factor of two, should be two-fold if a single cell type limits the response, four-fold if there are two interacting cell types both of which are limiting, and eight-fold, if there are three such limiting cell types. A plot of the log of number of added cells against log of the response will yield a slope of 1 for one limiting cell type, 2 for two limiting cell types, 3 for three limiting cell types, etc. In a large series of experiments, we found that the relationship between the log of number of primed nylon wool passed lymph node cells and the log of thymidine uptake in response to the immunizing antigen was 3. This suggests that the in vitro response normally involves the participation of three cell types. The nature of these interacting cells can be determined by adding a constant (and excess) number of a given cell type to all dilutions of the primed lymph node cells and measuring its effect on the slope of the resultant response. Using this analysis, it was shown that one of the cells was a radio-resistant cell present in spleens of non-primed mice; this cell is very likely an antigen-presenting cell. A second cell was a radio-sensitive T lymphocyte found in lymph nodes of primed and unprimed donors. This cell appears to represent a cell which is non-specifically "recruited" to proliferate. The third cell type appears to be the antigen-specific T lymphocyte. Analysis of histocompatibility restrictions and expression of immune response gene function in these systems are now in progress. These experiments provide an approach to determine the extent to which rules derived from "synthetic" systems apply to cellular interactions in unseparated lymphoid cell populations and will thus give a more complete understanding of the relative role of various cellular interactions in intact systems. (Tse, Schwartz and Paul; LI/NIAID).

Patients with Systemic Lupus Erythematosus have a Defect in Suppressor Cell Function Associated with a Serum Autoantibody

Systemic lupus erythematosus (SLE) is complex disorder associated with derangements in the regulation of immune responses and with the presence of antibodies to various autoantigens. Scientists in the Laboratory of Immunology and in the Arthritis and Rheumatism Branch of NIAMDD have shown that blood lymphocytes of SLE patients have a markedly diminished capacity to develop into suppressor cells as a result of stimulation with concanavalin a (Con A). Con A induced suppressor cells

from normal individuals will suppress in vitro proliferative responses to both pokeweed mitogen and autologous lymphoid cells (i.e. mixed lymphocyte responses [MLR]). In many patients with SLE, Con A fails to induce cells capable of suppressing either the pokeweed mitogen response or the MLR, whereas as other patients are defecient in only one of these suppressor functions. Sera from SLE patients with defects in capacity to develop suppressor cells contain cytotoxic antisera capable of causing complement mediated lysis of suppressor cell precursors in normal lymphocyte populations. Moreover, serum from patients with selective defects caused the same selective defect in normal lymphocyte populations treated with that serum and complement. These results suggest that separate T lymphocyte populations suppress the pokeweed mitogen response and the MLR. Fractionation of T lymphocytes into those possessing Fc receptors for IgG (T_γ cells) and those lacking such receptors indicates that T_γ cells preferentially suppress the MLR. These studies provide important insights into the pathophysiology of the immune response in SLE patients and help to expand our understanding of the normal regulation of the human immune response. (Sakane and Green, LI/NIAID; Steinberg, Arthritis and Rheumatism Branch, NIAMDD).

Lack of Role for the Fourth Component of Complement (C4) in In Vitro T Lymphocyte Activation

Other investigators have reported that antisera to human C4 inhibit the in vitro mixed lymphocyte response (MLR). This implies that C4, perhaps as a membrane molecule, may have a critical role in the response of T lymphocytes, either by acting on the stimulatory or responding cell. Because of the potential importance of this finding, Laboratory of Immunology scientists have carefully examined this issue in guinea pig systems. Antisera to C4, raised in guinea pigs, rabbits and goats, were found to have no effect on in vitro proliferative responses to antigens and mitogens and did not block the MLR. Furthermore, T lymphocytes from guinea pigs with a genetic defeciency in C4 (C4D) responded normally to each of these stimulants and cells of C4D guinea pigs had normal capacity to present antigens and to stimulate mixed lymphocyte responses. These results suggest that if C4 has an important role in the regulation of T lymphocyte activation, that role cannot be a general one involving all (or even most) types of such activation (Burger and Shevach, LI/NIAID).

Administrative, Organization and Other Changes

The Laboratory of Immunology plays an important role in the training of young scientists. During the past year, Drs. Theo Kirland, Virgil Woods, Jr., Loran Clement and Ronald Wilder completed their appointments as research associates. Dr. Constantin Bona, who was a Visiting Scientist; Drs. Bondada Subbarao, Tsuyoshi Sakane and Franc Meloni, who were Visitng Fellows; and Drs. Herbert Herscowitz, Ole Werdelin, Kim Bottomly and Harley Tse, who were Guest Workers, completed research and training programs in the Laboratory of Immunology. Margaret Simons received her Ph.D. in genetics for research carried out in the Laboratory under the supervision of Dr. Rose Mage. Each of these scientists made substantial research contributions during their tenure in the Laboratory of Immunology.

Drs. Laurie Glimcher, Dan Hansburg, John Schmidt and David Cohen entered the Laboratory as research associates while Dr. Dan Longo was converted from a Guest Worker to a Research Associate. Drs. Louis Matis and Robert Clark, clinical associates in the Laboratory of Clinical Investigation, were assigned to the Laboratory of Immunology. Drs. John Kung, Joe Chiba, Benjamin Sredni, and Shunichi Kumagai were appointed as Visiting Fellows and Dr. Leona Fitzmaurice entered the Laboratory as an Expert Consultant.

Finally, one of the senior staff of the Laboratory, Dr. Donald Mosier, resigned his position in order to accept a major appointment at the Institute for Cancer Research, Fox Chase, Pennsylvania. Dr. Mosier had established an internationally recognized research program in cellular immunology. His loss as a contributor to the Laboratory program will be deeply felt.

Honors, Awards, and Scientific Recognition

Laboratory scientists play important roles in the national and international scientific community. Many serve on editorial boards of important journals. Dr. Paul was appointed to the editorial board of the Journal of Immunology; Drs. Schwartz and Shevach are associate editors of this journal. Dr. Green and Dr. Shevach are referee editors of the Proceedings of the Society for Experimental Biology and Medicine. Dr. Mage is chairman of the editorial board of Federation Proceedings and is a member of the editorial board of the Journal of Immunologic Methods. In addition, Dr. Paul is an advisory editor of the Journal of Experimental Medicine and an associate editor of Immunologic Reviews, The Scandinavian Journal of Immunology, Immunologic Communications, CRC Critical Reviews in Immunology, and Human Immunology. Dr. Green is an associate editor of Clinical Immunology and Immunopathology, and Dr. Inman is an advisory editor of Molecular Immunology. Ms. Lieberman completed a term as an advisory editor of that journal. Dr. Shevach is a member of the editorial advisory board of the FASEB Handbook on Inbred Strains of Laboratory Animals.

Dr. Paul presented invited lectures at the Southeastern Immunology Workshop, the Armand Hammer Conference on the Regulation of the Immune Response, the Midwest Student Medical Research Forum, the Scottsdale Conference on B Lymphocytes in the Immune Response, the Jane Coffin Childs Symposium, the Fogarty Center Workshop on Mediation of Effector Functions by Antibodies and the Symposium on Carcinogeneses of the Given Institute. He served as summarizing speaker at the National Institute on Aging Conference on Immunology and at the International Symposium on Aging and Immunity and was a session chariman at the Brook Lodge Conference on Mononuclear Phagocytes and the Scottsdale Conference on B Lymphocytes in the Immune Response. In addition Dr. Paul was elected President-elect of the American Society for Clinical Investigation and was appointed chairman of the American Association of Immunologists Program Committee. He is a member of The American Cancer Society Committee on Personnel for Research and the Arthritis Foundation Fellowships sub-committee and is co-organizer of the Cold Spring Harbor Immunogenetics Course.

Dr. Green was an invited lecturer at the Intenational Congress on Systemic Lupus Erythematosus, held in Kyoto; at the International Congress on Vasculitis in Innsbruck; and at the Summer Course in Methods of Immunological Research and Diagnosis. He is co-chairmen of the American Association of Immunologists Program Committee and is a member of the Transplantation Immunology Committee of NIAID.

Dr. Shevach gave major lectures at the ICN-UCLA meeting on T and B Lymphocytes, at the Annual Meeting of the New England Society for Allergy and at the Brook Lodge Conference on Mononuclear Phagocytes. He is a member of the Allergy and Immunology Study Section of the Division of Research Grants, NIH.

Dr. Schwartz gave invited lectures at the ICN-UCLA Meeting on T and B Lymphocytes and at the Brook Lodge Conference on Mononuclear Phagocytes. He was co-chairmen of a minisymposium at the annual meeting of the American Association of Immunologists.

Dr. Mage, Ms. Lieberman and Dr. Bona were invited lecturers at the International Colloquium in honor of Jacques Oudin at the Institut Pasteur, in Paris. Dr. Mage is Vice-President of the D.C. Chapter of the Society of the Sigma Xi. Ms. Lieberman is a member of the American Association of Immunologists Membership Committee.

Dr. Inman was an invited speaker at the European Molecular Biology Organization Workshop on Accuracy in Biology, at the Netherlands Red Cross Transfusion Service and at the Central Laboritorium TNO Ryswegh, Netherlands. Dr. Waxdal was a major speaker at the Colloquium on Membrane Glycoconjugates, in Seillac, France, and at the Colloquium on Protides of the Biological Fluids, in Brussels. He was an invited discussion leader at the Symposium on Biomedical Applications of the Horseshoe Crab at Woodshole MA. and was chairman of the session on Biochemical Consequences of Mitogen Activation at the annual meeting of the American Association of Immunologists.

In addition, laboratory scientists presented research seminars at major universities and research institutes in the United States and abroad.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00030-11 LI

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Antigen Recognition and Activation of Immunocompetent Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: William E. Paul, Chief, LI/NIAID

Other Investigators: Rose Lieberman, Sr. Investigator, LI/NIAID
Donna G. Sieckmann, Staff Fellow, LI/NIAID
Kathryn Stein, Staff Fellow, LI/NIAID
Toby Hecht, Sr. Staff Fellow, LI/NIAID
Constantin Bona, Visiting Scientist, LI/NIAID
Patricia Mongini, Guest Worker, LI/NIAID
John Kung, Visiting Fellow, LI/NIAID
Ellen Heber-Katz, Staff Fellow, LI/NIAID
James Mond, Uniformed Services University of the Health Sciences
Irwin Scher, Naval Med. Res. Inst.
Aftab Ahmed, Naval Med. Res. Inst.
Steven Kessler, Naval Med. Research Institute
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COOPERATING UNITS (if any)
LMI/NIAID; Naval Medical Research Institute; Institute for Cancer Research, Philadelphia, PA

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SECTION

INSTITUTE AND LOCATION National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
9	6	3

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The general goal of this project is to delineate the mechanisms involved in antigen recognition by and activation of thymus-independent (B) and thymus-dependent (T) lymphocytes. During the past year our efforts have concentrated on: 1) the development of new genetic models of B lymphocyte deficiency; 2) the identification and genetic mapping of B lymphocyte differentiation antigens; 3) the study of genetics of IgD and of recombination within the IgC_H gene complex; 4) the genetic regulation and chemical characteristics of antibodies produced in response to polysaccharide haptens; 5) the regulation of antibodies and T lymphocytes capable of interacting with membrane immunoglobulin. In addition, studies of the regulation of growth of vesicular stomatitis virus in spleen cells, both in vivo and in vitro are underway.

PHS-6040
(Rev. 10-76)

Genetic models for the study of B lymphocyte development

Over the past several years we have investigated the developmental and functional heterogeneity of thymus-independent (B) lymphocytes through the use of a mutant mouse strain. Mice of this strain, CBA/N, are unresponsive to thymus-independent type 2 (TI-2) antigens and lack a subset of B lymphocytes which express the differentiation antigens Lyb 3,5, and 7. Recently, we have observed that breeding the mutant CBA/N gene, xid, on to a C3H background leads to a much more profound defect due to a genetic synergy between the xid gene and one or more C3H genes. The "synergistic defect" (SD) is characterized by in vitro unresponsiveness to TI-1 as well as TI-2 antigens and by a failure of B lymphocytes to proliferate in response to a series of mitogens including lipopolysaccharide, bacterial lipoprotein, and Nocardia water soluble mitogen. Mice with the SD phenotype have diminished numbers of B lymphocytes and the B lymphocytes they possess express very low ratios of membrane (m) IgD:mIgM. We are currently in the process of preparing inbred and congenic mouse strains expressing the SD phenotype in order to make these mice available for the detailed study of B lymphocyte function and development.

A concurrent program is the analysis of B lymphocyte subsets through the development of antisera to differentiation antigens expressed by these subpopulations. In last year's report, we described the identification and mapping of Lyb 5 and Lyb 7. Both markers are found on that subpopulation of B lymphocytes which is lacking in mice with the CBA/N immune defect. Lyb 7 is particularly interesting because antisera to it block B lymphocyte activation by TI-2, but not TI-1, antigens and because Lyb 7 is genetically linked to genes specifying immunoglobulin heavy chain constant regions (IgC_H genes).

We are attempting to expand our "library" of antibodies to B lymphocyte differentiation antigens by the preparation of rat anti-mouse B lymphocyte hybridomas. A series of such hybrid tumors have been produced, and preliminary analysis of their secreted products is in progress. Several of these reagents appear to identify B lymphocyte subpopulations and will be of considerable interest in our continued study of B lymphocyte function.

Linkage and recombination of immunoglobulin structural genes.

Studies of the genetic regulation of mIg expression are also in progress. We have available a series of monoclonal, allo- and hetero- anti-δ antibodies and have recently completed an extensive strain survey allowing us to more completely make IgC_H haplotype assignments among common inbred laboratory strains. An interesting result of this is the observation that the d and e haplotype groups need to be further subdivided because strains previously assigned to each of these groups differ in IgD allotype from other strains within the same group. On this basis, we have defined the n and o IgC_H haplotypes. Furthermore, one inbred strain, AL/N, appears to be a natural IgC_H recombinant between the A and AKR strains, possessing the IgD alleles of A and the IgG alleles of AKR. This observation is supported by our identification of two actual intra-IgC_H recombinant mice, which arose

during backcrossing of (C57BL/6 x DBA/2)F_1 hybrids to C57BL/6. These mice, individuals 72 and 74, express mIgD and serum IgD of the DBA/2 type but lack DBA/2 type IgM, IgG_1, and IgG_{2a}. This phenotype is stable on subsequent breeding of 72 and 74 to C57BL/6. Furthermore, when mice of the 72 and 74 families are crossed to strain A mice, individuals which inherit DBA/2 IgD also inherit C57BL/6 IgG_{2a}, proving that these allelic genes are on the same chromosome and establishing that a recombination has actually occurred. The availability of these IgM-IgD recombinant strains should make possible the precise mapping of genes for V region markers with reference to these two critical constant region genes.

Genetics of immune responses

In association with these studies of genetics of immunoglobulin structural genes, studies of the genetic basis of antibody responses to highly defined polysaccharide antigens are in progress. Lieberman and her colleagues have previously established that the capacity to make antibodies to β(2→1) fructosan determinants, such as those in inulin, upon immunization with bacterial levan, a β (2→6) polyfructosan with β(2→1) branch points, is determined by genes linked to IgC_H genes. Our current studies indicate that non-IgC_H genes play a critical role in the magnitude and clonal diversity of the response. Most strikingly, BALB/c mice immunized with bacterial levan make a highly restricted anti-inulin response, dominated by an idiotype-positive antibody appearing as a characteristic triplet of bands on isoelectric focusing. Although C57BL/6 mice lack the structural genes for the triplet and related anti-inulin antibodies, they possess non-immunoglobulin genes which increase and diversify the response. This is shown by an analysis of responses of (BALB/c x C57BL/6)F_1 hybrids, allotype congenic B.C8 mice, and recombinant inbred CXB mice. Our current analyses suggests that two distinct genes are involved; we are in the process of verifying this and mapping those genes. In addition, we are studying the genetic regulation of the response to isomaltotriose and isomaltohexose derivatives of proteins in normal mice and in mice with the CBA/N immune defect.

Regulation of B lymphocyte activation through mIg

Over the last several years, we have developed persuasive evidence that purified antibodies to IgM and to L chains can stimulate substantial proliferative responses on the part of B lymphocytes while, at the same time, inhibiting the secretion of immunoglobulin in response to other stimulants. Our data has indicated that this proliferative response does not require the presence of either T lymphocytes or macrophages but that it is a function of a late appearing subset of B lymphocytes which is absent in mice with the CBA/N immune defect. During the past year, a detailed study of the phenotype of the responding cells has been carried out. Particular emphasis has been placed on the characterization of these cells in terms of the density of mIg of various classes expressed on their membranes. To carry out these studies, B lymphocytes have been prepared and separated into subpopulations with distinct amounts of Ig on their surface through the use of the fluorescence activated cell sorter. Through this approach, we have

shown that cells which proliferate in response to both anti-μ and anti-κ express a high density of total Ig, a low density of IgM, and an intermediate to high density of IgD. Preliminary studies indicate these cells also express Lyb 5. We are now in the process of preparing hybridoma rat anti-mouse μ antibodies inorder to more decisively explore the recognition events involved in B cell activation.

In a closely related set of studies, we have shown that T lymphocytes with receptors specific for idiotypic determinants found on the TNP-binding myeloma protein MOPC460 suppress the production of anti-TNP antibodies which express this idiotype. Furthermore, we have evidence that such cells not only exist but that they play an important normal role in regulating the clonal nature of the anti-TNP response to TNP antigens. These studies are particularly interesting because they provide one of the first examples of direct action of suppressor T cells on B cells and because they comprise one of the first instances of the physiologic activity of the idiotype-anti-idiotype network.

Regulation of infection of spleen cells with vesicular stomatitis virus (VSV)

VSV is a membrane budding virus which fails to grow in resting lymphocytes and which has been reported to contain host cell membrane proteins, such as H-2 antigens, in its envelope. We are in the process of testing the thesis that H-2 antigens associated with VSV virions may provide a means of attack of the host immune system upon virus and that H-2 antigens associated with virus play a strong evolutionary role in promoting H-2 diversity and the high level of "spontaneous" immunity to H-2. To study this, we have developed procedures for the in vivo and in vitro infection of mouse spleen cells with VSV obtained from various cell lines and have shown that immunity to the cell line in which VSV is grown profoundly effects the capacity of the virus to infect spleen cells in vivo or in vitro. Studies are in progress to obtain more reproducible conditions for inducing in vivo and in vitro infection of lymphocytes by VSV, for more precisely evaluating the immunologic mechanisms underlying the distinctions made between VSVs obtained from cell lines of distinct H-2 type, and to directly determine what host component is, in fact, responsible for the capacity of the immune system to determine the "lineage" of the virus.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AI-00148-04 LI

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Lymphocyte Interactions, Receptors and Functions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: Ira Green, M.D., Senior Investigator, LI/NIAID

Other Investigators: Tsuyoshi Sakane, LI/NIAID
Myron Waxdal, LI/NIAID
Ethan Shevach, LI/NIAID
Alfred Steinberg, Arthritis & Rheumatism Branch, NIAMDD/NIH
Herbert Herscowitz, Dept. of Microbiology, Georgetown University School of Medicine

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SECTION

INSTITUTE AND LOCATION National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	2	2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A B cell leukemia (L_2C) of inbred strain 2 guinea pigs and a new myelogenous leukemia (G-13) of inbred strain 13 guinea pigs are being investigated as models of human leukemia. The chemical nature of the tumor specific transplantation antigen (TSTA) of the L_2C leukemia is being investigated by immunization protection tests in syngeneic animals. The TSTA of the L_2C leukemia is unusual in that it is of low molecular weight and is extremely resistant to heating. The G-13 leukemia is being examined for the presence of a TSTA and the presence of cell surface markers. As in the human, the myeloblasts of this leukemia are Ia^+. The immune function of these cells are also being investigated.

Patients with systemic lupus erythematosus (SLE) have a defect in the development of Con A induced suppressor cells. Moreover, the sera from lupus patients (more particularly the IgM fraction) can induce suppressor cell defects in populations of normal lymphocytes. T_γ and $T_{non\gamma}$ cells mediate different types of suppression; certain lupus sera are specifically cytotoxic for T_γ cells. Finally T cells able to proliferate in response to autologous non T^γ cells act as precursors of Con A induced supppressor cells.

PHS-6040
(Rev. 10-76)

I. Studies of the Guinea Pig L_2C Leukemia and a New Myelogenous Leukemia

The L_2C lymphatic leukemia of inbred strain 2 guinea pigs arose about 25 years ago in an old female strain 2 guinea pig. Studies in our laboratory starting 8 years ago have shown that this leukemia cell is of B cell origin and has surface IgM and C3 receptors. There are several variants of this leukemia; one variant exists which lacks Ia antigens (an antigen of the major histocompatibility complex of the guinea pig). Our laboratory has demonstrated that, as measured by immunization protection tests in inbred strain 2 guinea pigs, the L_2C cell has a powerful tumor specific transplantation antigen (TSTA). However, in the Ia negative variant of the L_2C cell, this TSTA is not immunogenic. Our previous studies suggested that the TSTA is not related to the surface IgM molecule.

The main goal of the present study is to identify the chemical nature of the TSTA of the L_2C leukemia cell. A 3M KCl extract of the L_2C cell contains the TSTA as measured by immunization protection tests in syngeneic strain 2 animals. Immunization with as little as 100 µg of this material per animal confers protection against a lethal challenge of L_2C cells. Fractionation of the KCl extract on Sephadex G-200, DEAE cellulose and CM cellulose indicate that the immunogenic molecule has a M.W. of less than 20,000 and is basic. The material is very resistant to heat and extremes of pH but is destroyed by treatment with trypsin, neuramnidase and periodate. These properties suggest that the TSTA is a low m.w. glycoprotein. Attempts to further fractionate the material by polyacrylamide gel electrophoresis were unsuccessful, none of the fractions eluted from these gels were immunogenic.

Most recent studies have used CM cellulose followed by G-50 chromatography. Several immunogenic regions from G-50 chromatography have been obtained and are now being currently investigated. Progress in the chemical identification of the TSTA of the L_2C cell has been slow because each individual assay takes 7-8 weeks to complete (in vivo protection being the final end point).

In addition to the studies of the L_2C leukemia, we have been studying a new myelogenous leukemia of strain 13 guinea pigs induced with the carcinogen N-nitroso-N-butylurea (Evans et al., Cancer Research, Vol. 38: 130, 1978). This leukemia is serially transplantable in inbred strain 13 guinea pigs using intradermal or intraperitoneal injection of whole cells. Two to three weeks after injection of the cells there is a rise in polymorphonuclear leukocytes; this is soon followed by an increased number of myeloblasts. The WBC finally rises to > 100,000 cmm and the animal succumbs. Paralysis of the hind legs is often observed as a terminal event.

We have characterized the surface markers on these myeloblasts; these cells lack surface Ig and do not have C3 or Fc receptors. These cells do have Ia antigens on their surface as determined by immunofluorescent techniques; biosynthetic studies indicate that the Ia antigens found on these cells are actually synthesized by the cells.

To determine whether Ia antigens were also found on more mature cells of the myelocytic series the cells were first stained in suspension in the living state for the presence of surface Ia antigens. The cells were then smeared on gridded slides (each grid having a number) and the position of the Ia positive (stained) cells was noted. The smear was then stained by Giemsa stain, the same cells were again located and their morphology noted. The results of such experiments demonstrated that most myeloblasts were Ia^+ whereas most bands and polymorphonuclear leukocytes were negative.

The Ia positive myeloblasts were then studied for two immunological functions. First the myeloblasts were tested for their ability to act as stimulator cells for the MLR. Second the myeloblasts were tested for their ability to act as antigen presenting cells for the T cell proliferative response to antigen. They failed in both of these functional tests. This observation strongly suggests that the presence of surface Ia antigens is necessary but not sufficient to allow a cell to perform such immunological functions.

Preliminary immunization protection studies to determine whether these cells possess a tumor specific transplantation antigen (TSTA) indicate that the cells do have a TSTA. In immunized animals, large fungating tumor masses were observed to regress.

Significance to Biomedical Research and the Program of the Institute

The properties of the TSTA of the L_2C leukemia cell are highly unusual and differ from the properties of most other TSTA described by other investigators. Most other TSTA have a m.w. greater than 40,000 daltons and are sensitive to heat.

The fact that this TSTA is on a malignant B lymphocyte is of particular interest; once the L_2C TSTA is more completely identified one could look for a similar TSTA in several different human B cell leukemias. Another possibility is that this TSTA could represent a marker for a particular stage of B cell differentiation present on only a very small number of normal B cells.

Studies of this myelogenous leukemia are of potentially great interest since in some cases of human myeloid leukemia the myeloblasts are also Ia positive. The availability of large numbers of Ia^+ myeloblasts may make possible further chemical studies of these Ia antigens and to allow comparison of these Ia antigens with those on lymphoid cells. Further functional studies using these Ia^+ myeloblasts may provide a clue as to why Ia antigens are present on myeloblasts. This leukemia may also serve as a model for chemo-immunotherapy of myelogenous leukemia.

Human Suppressor Cells in Health and Disease

The second aspect of our studies concerns suppressor cells in patient with systemic lupus erythematosus (SLE). The system we are using is adapted from Shou et al., J. Exp. Med. 143, 1100, 1976. The use of this system to

describe concanavalin induced suppressor cells in normal individuals has already been published by us in the J. of Immunology 119, 1169, 1977.

A two stage assay system is employed in which T lymphocytes are exposed to concanavalin A (Con A) for 3 days to induce suppressor function and then these cells, after mitomycin C treatment, are added to an assay system consisting of lymphoid cells obtained from the same donor 3 days later. As a control, another aliquot of cells incubated without Con A is used. These assay cultures are then stimulated by either mitogen or allogeneic cells, cells from the first culture are added and the degree of proliferation is measured by ^{3}H-thymidine incorporation.

We have recently demonstrated that aliquots of these same Con A activated T cells can also suppress a pokeweed mitogen (PWM) induced polyclonal B cell response.

Our major effort has been to analyze suppressor cell defects in patients with SLE. We first demonstrated that patients with SLE have a relative lack of T cells capable of being induced to form suppressor cells. Furthermore when certain SLE sera are added to cultures of normal lymphocytes during the Con A mediated induction of suppressor cells, no suppressor cells develop. We had previously shown that the IgM fraction of SLE sera was responsible for this effect and that complement is necessary. Our most recent studies have revealed that the SLE sera is only effective when added at the beginning of the Con A inducing culture but not at the end. We also observed that certain patients with SLE had a selective loss of only one type of suppressor cell. That is, the patients lymphocytes could suppress the PWM proliferative response but not the MLR. The sera from these same patients could induce in an in vitro culture exactly this same pattern of defects in the lymphocytes from normal individuals. The fine cytotoxic specificity of the sera from these selected patients was then tested on different classes of T cells. T cells can be separated into two classes, one class having a surface receptor for the Fc portion of IgG. (T_{γ} cells) and another class lacking this receptor ($T_{non\gamma}$ cell). These sera were selectively cytotoxic for T_{γ} cells. We then investigated whether different T cell classes could differentially suppress either the PWM proliferative response or the mixed lymphocyte response (MLR). We observed that T_{γ} cells could preferentially suppress the MLR. Thus the ability of certain SLE sera to eliminate suppressor cells for the MLR can be ascribed to their preferential cytotoxicity for T_{γ} cells.

We have also performed two other related studies. First we have serially studied patients with SLE during the active and inactive phases of their disease for both defects in suppressor cells development for defects in the autologous MLR (discussed in last year's annual report).

In each case these in vitro tests were abnormal during the active phase of disease and returned to normal when the disease activity decreased. These results strongly suggest that the abnormalities of suppressor cell generation and the autologous MLR observed in lymphocytes from patients with SLE are not intrinsic to the lymphocyte or genetically determined.

In the second study we wished to determine whether cells proliferating in the autologous MLR were particularly able to develop Con A induced suppressor function as compared to cells proliferating in the usual cells MLR. Therefore cells undergoing either auto MLR or allogeneic MLR were treated or not treated with BUdR and light.

The T cells were then harvested and tested for their ability to generate suppressor function. The cells undergoing an autologous MLR in the presence of BUdR and light failed to subsequently develop suppressor function whereas the cells undergoing an allogeneic MLR were able to develop suppressor function.

Significance to Biomedical Research and the Program of the Institute

The defect in the development of suppressor cells in patients with SLE may be one of the causes of increased auto antibody production in this disease. Furthermore, sera from patients with SLE can induce suppressor cell abnormalities in normal T lymphocyte. Strategies to increase suppressor function in patients with SLE might have therapeutic potential. Selective removal of antibodies to suppressor cells might be a step in this direction.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00035-06 LI

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Specificity in Immune Responses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: John K. Inman, Senior Investigator, LI/NIAID

Other Investigators: Dr. Ettore Appella, LCB/NCI
Dr. Garrett C. DuBois, LCB/NCI
Dr. Bruce Merchant, BOB/FDA

COOPERATING UNITS (if any)
Laboratory of Cell Biology, National Cancer Institute; Immunology Branch, National Cancer Institute; Bureau of Biologics, FDA.

LAB/BRANCH
Laboratory of Immunology

SECTION

INSTITUTE AND LOCATION National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long range objective of this project is to carefully re-examine the nature of binding specificity at antibody combining regions and other types of receptor sites. The effects of polyvalent binding on binding energy and specificity will be considered, and together these properties will be related to the behavior of immune systems. In particular, multispecific binding of disparate structures by individual antibody species is being studied through screening of specifically purified, radiolabeled antibodies with affinity chromatography columns. The ligands are bound to these columns using large haptenic reagents synthesized specially for this purpose using methods of peptide synthesis. Multispecific binding will be used for producing and isolating homogeneous antibody by cross-stimulation and immunoadsorption. A number of chemical techniques and products that have been developed from the above work are being used in collaborative studies on molecular interpretation of cellular mechanisms in immune responses. Materials employed include synthetic thymus-independent antigens.

PHS-6040
(Rev. 10-76)

Project Description

Objectives

The overall objective of the project is to gain more complete understanding of the molecular basis for specificity in immune responses. The binding properties of antibodies and other receptor-bearing molecules are to be studied and related to the behavior of immune systems. Specific objectives are the following:

1. To assess the generality and frequency of multispecific binding by individual antibody combining regions by means of screening experiments employing many diverse, structurally unrelated haptens.

2. To develop methodology required for such screening tests including the synthesis of numerous, large haptenic reagents.

3. To employ multispecific binding and cross-stimulation in raising high titers of highly restricted antibodies for fine specificity studies and for use as highly specific reagents in immunological studies.

4. To use the large hapten screen to find disparate structure cross-reactions useful in identifying antibody-secreting cell clones in studies assessing antibody diversity.

5. To use the screen in studying the multispecific character of other types of receptors and to employ observed cross-reactions in a general approach to affinity separations of receptors.

6. To employ methods and reagents developed in this project in collaborative studies dealing with specificity in immune responses.

Major Findings

The main emphasis during the past year has been placed on exploring strategies for the efficient synthesis of highly varied specificity probes. These probes are reagents for introducing large haptenic groups onto macromolecular carriers, and they play a key role in fulfilling the above research objectives. With the collaboration of a postdoctoral fellow, Dr. Suresh Shukla, and my assistant, Miss Barbara Duntley, I have evaluated a large number of synthetic intermediates, pathways, tactics and strategies leading to branched peptide derivatives bearing a variety of substituent structures and attachment functions. Our findings are as follows: the use of glutamic acid as a trivalent hub structure sometimes led to undesired by-products when the last substituent was introducted. This result seemed to preclude, for the time being, the glutamic diamide approach as being sufficiently general and facile to meet the demands of our study. Instead, we turned our attention mainly to another trivalent amino acid, L-lysine. We have successfully synthesized large haptenic reagents having attachment functions and spacing structures connected with either the alpha-carboxyl or

epsilon-amino group. About 20 of the latter type of reagent have been prepared in final form and are at present undergoing purification. Techniques for achieving final purity are being studied. We have explored adipic acid as a spacer for the attachment function on lysine and have concluded that the latter should be a protected hydrazide introduced at an early stage of synthesis. In the course of this work we introduced a new protecting group for acyl hydrazides, the 2-(methylsulfonyl)ethoxycarbonyl (Msc) function which is stable during the removal of other blocking groups. It can be removed under special, mild conditions just prior to activation and coupling of the hapten to a carrier (immunogen or adsorbent matrix). The Msc group has not, to our knowledge, been used for hydrazide protection. This finding should have general application in peptide synthesis: Along with our experience in haptenic reagent synthesis, it was presented in a poster paper at the Sixth American Peptide Symposium on June 21, 1979. Currently we are evaluating haptens based on L-ornithine as a central structure. A number of substituent structures have been selected or prepared for the next series of probes. Enough haptenic reagents are on hand to begin setting up a preliminary screen for multispecific binding; preparations are underway.

Work on sequencing chemistry has largely been concluded. I am presently writing a chapter (with Dr. Appella) summarizing our thoughts and experiences over the past few years on the topic of newer approaches to solid- and liquid-phase amino acid sequence analysis.

In collaboration with Dr. Bruce Merchant (BOB/FDA) and Dr. Harm Snippe (State University at Utrecht, The Netherlands) several lines of investigation were continued, and results are currently being submitted for publication. It was found earlier that immunization of mice with various large haptens, conjugated to bovine serum albumin and mixed with the cationic, surface-active lipid, dimethyl dioctadecyl ammonium bromide (DDA), generated delayed type hypersensitivity (DA) without detectable levels of circulating specific antibody. DDA promoted strong cross-reactivity with other similar, and not so similar, haptens on the same carrier. Both direct and indirect plaque-forming cells (PFC) were detected, however, in peripheral lymph nodes and spleens 4 days after a challenge injection. The specificity of the PFC was distinctly greater than that exhibited by DH. Another area of collaborative work with Drs. Merchant and Snippe concerns the nature of the X-linked, B-cell defect of CBA/N mice. We had earlier reported that the essentially normal immune response of these animals to thymic dependent, hapten-carrier conjugates can be blocked by very small amounts of the same hapten bound to a thymic independent carrier such as Ficoll or SIII polysaccharide. The blockade was presumed to be mediated in part through efficient binding of the blockading antigen by Ig receptors on the defective B cells. A subsequent study supported this view: normal CBA/N x C3H/HeN F_1 female spleen cells when exposed to DNP-AGG-Ficoll in vitro could establish a specific PFC response after transfer into irradiated, male, hybrid recipients. Although defective male, hybrid spleen cells could not mount this rescue function, they could convey immunogenic quantities of DNP-AGG-Ficoll, previously bound to their surfaces, to female cells in vitro This conveyor function was impeded by pretreating the male cells with goat

anti-mouse μ serum. The defective cells thus appear to bind thymus-independent antigens but not to be triggered by the binding event. Additional experiments with cell transfer systems using CBA/N x C3H/He F_1 hybrid spleen cells and studying the effects of the blockading antigen, DNP-AGG-Ficoll, showed a different pattern of blockade than was observed in in vivo trials: Direct and indirect PFC responses, upon secondary immunization in irradiated recipients, were subject to hapten-specific blockade; under some cell transfer conditions normal CBA x C3H/HeN F_1 female spleen cells were just as susceptible to blockade as the defective male hybrid cells.

Significance to Biomedical Research and the Program of the Institute:

An essential characteristic of many immune mechanisms is the specificity of action which is directed by definitive chemical structures. An understanding of specificity in immune systems rests first upon an adequate knowledge of the scope and nature of specificity at the level of single receptor-determinant interactions. Therefore, a principal aim of this project is to carry out the first sytematic and general exploration of the phenomenon of multispecific binding. Because of the diversity and availability of antibodies, these receptor-bearing molecules provide ideal models for such studies in addition to their being interesting as important components of many types of immune reactions.

Cooperative studies have proven the value of large hatpenic reagents and other synthetic or semi-synthetic antigenic components in exploring specificity as it is manifested in more complex, multi-receptor-determinant interactions. Reagents or reactions developed for the explicit requirements of this project can have interesting applications in many basic studies in immunology such as those aimed at elucidating the mechanisms of B and T cell specific activation.

Future Course:

The screening for multispecific binding will commence in the next month. Early studies will involve some monoclonal antibodies (myeloma and hybridoma proteins) and some selected antisera. Synthesis of large haptenic reagents will continue on a more-or-less routine basis in order to enlarge the repertoire of our screen. A large number of hybridoma antibodies will be sought (as gifts from other laboratories) and used for study. Animals will soon be immunized against our large haptens for production of normal heterogeneous antibodies and possibly for production of hybridomas (as a collaboration within LI/NIAID). Collaborative work with Dr. Merchant will continue.

PUBLICATIONS:

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Inman, J.K.: Peptide synthesis with minimal protection of side-chain functions. In Gross, E. and Meienhofer, J. (Eds): The Peptides-Analysis, Synthesis and Biology, Vol. 3, N.Y., Academic Press, Inc., in press.

HONORS:

Appointed as Advisory Editor to the Journal, Molecular Immunology.

Invited speaker at The European Molecular Biology Organization, Workshop on Accuracy. Grignon, France, September 1978.

Invited lecturer at the State University at Utrecht, The Netherlands, September, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI-00036-14 LI

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Immunoglobulin Genetics: Regulation of Gene Expression and Lymphoid Differentiation.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: Rose G. Mage, Ph.D., Senior Investigator, LI/NIAID

Other Investigators: G.O. Young-Cooper, LI/NIAID
C.B. Alexander, LI/NIAID
R. Wilder, Research Associate, LI/NIAID
H. Abdi, Visiting Fellow, LI/NIAID

COOPERATING UNITS (if any) M.D. Cooper and A.R. Lawton, Univ. Alabama, Birmingham, AL: J. Karsh, A.R./NIAMDD; I. Scher, NMRI; A. Chersi, Istituto Regina Elena, Rome, Italy; E. Appella, LCB/NCI; R. Asofsky, LMI/NIAID. In addition, we cooperate with a large number of investigators at NIH and elsewhere by supplying research reagents and allotype-defined rabbits and by providing typing information about rabbit sera sent to us.

LAB/BRANCH
Laboratory of Immunology

SECTION

INSTITUTE AND LOCATION National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20205.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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SUMMARY OF WORK (200 words or less - underline keywords)

Overall goals of this project are to define the nature of genes which code for, or otherwise affect the structure of immunoglobulins(Igs) and to understand the processes which regulate Ig-gene expression and which lead to synthesis of highly specific antibody molecules. We prepare immunological reagents which detect structural differences between genetically controlled polymorphic forms of rabbit Igs (Ig allotypes). We use immunological, chemical and molecular genetic approaches to define the Ig-structural differences and to learn more about the chromosomal organization of Ig genes, and the regulated expression of antibodies and antigen-specific surface receptors on lymphocytes. Inheritance patterns and the levels of regulation of phenotypic expression of alleles and chain types are investigated by breeding experiments, by studies of ontogeny and differentiation of cells of the rabbit immune system and by direct molecular genetic analyses. Two major new efforts which are planned will involve the use of recombinant DNA molecules to: 1) examine genome organization by isolation of rabbit Ig-genes and analysis of variable and constant region DNA sequences as well as adjacent sequences; and 2) identify nuclear RNA precursors and their processing steps in the production of immunoglobulin mRNA. 22-30

Objectives

The objectives of this research program are to define in molecular terms, the organization and regulated expression of rabbit immunoglobulin genes, the levels of regulation of lymphoid cell differentiation, and the mechanisms which lead to the synthesis of highly specific antibody molecules and antigen specific lymphocyte cell surface receptors.

Methods Employed

For the studies, we prepare and characterize antisera which detect structural differences between genetically controlled polymorphic forms of rabbit Igs (Ig allotypes) and between classes and subclasses, types and subtypes of their polypeptide chains. We use immunological and chemical approaches to define structural differences and conduct classical and molecular genetic experiments to learn more about the chromosomal organization of genetic information related to Ig molecules. Problems which until recently have been approached at the levels of serology and classical Mendelian genetics are now amenable to direct molecular genetic analysis. We are initiating studies which will utilize characterized clones containing mouse immunoglobulin heavy and light chain sequences as well as efforts to clone rabbit genomic DNA and complementary DNA copies of mRNA so that we can examine the organization of rabbit immunoglobulin genes and identify nuclear RNA precursors and the processing steps in production of immunoglobulin mRNA.

Major Findings

Ontogeny, lymphoid cell differentiation, and the regulation of Ig-gene expression.
Detection of V_H and studies of allelic-exclusion in pre-B cells.

In previous studies, we described a primitive lymphoid cell found in fetal liver and in the bone marrow of older rabbits which contained cytoplasmic IgM but lacked surface IgM detectable by immunofluorescence (Hayward et al.,1978). In heterozygous b^4b^5 rabbits, the pre-B cells in which we could detect these kappa chain allotypes appeared to exhibit allelic exclusion. Comparable studies have now been done (Gathings, Cooper, Lawton, Young-Cooper and Mage--work in progress) with the allotypes associated with the variable regions of immunoglobulin heavy chains. Affinity-purified and cross-absorbed a1 anti-a2 and a1 anti-a3 reagents were used to identify V_H-bearing immunoglobulin within pre-B cells of a2, a3 and heterozygous a^2a^3 rabbits. We found that essentially all pre-B cells from adult and newborn heterozygous animals which are detectable with our fluorescent reagents exhibit allelic exclusion of the a allotypes. The majority of cells with cytoplasmic IgM have detectable V_Ha allotypes (56-100%). We have also investigated B cells with the reagents described above (Gathings et al.) as well as with a2 anti-a3 and a3 anti-a2 reagents (Wilder, Abdi, Scher and Mage, in progress). In the latter studies, analytical flow microfluorometry (FACS analysis) was performed on fluorescein labeled cells rather than doubly stained cells. Doubly heterozygous rabbits

were used and single populations were scored for proportions of total Ig-, b4-, b5-, a2-, and a3-positive cells. The proportions of Ig-positive were compared to the sums of a2 + a3 positive and b4 + b5 positive cells. No evidence for B cells expressing two allelic types was obtained by either the double staining or the FACS technique. Cells tentatively identified as myeloid or pre-myeloid cells were found in adults which appear to bind Ig of both allotypes in heterozygotes, presumably through high avidity Fc-receptors (the cells still stain for both allotypes after overnight incubation at 37°C) (Gathings et al). Some reports of "allelic inclusion" from other laboratories may be explained by scoring of such cells. We are currently comparing the results of analysis of surface Ig allotypes on peripheral blood lymphocytes detectable by FACS analysis and by a specific rosetting assay because some of the reports of "doubles" utilized rosetting assays (Abdi, Scher and Mage, in progress). Reagents have been prepared and their specificity demonstrated so that additional studies (in progress) can determine whether imbalances in allelic allotype expression which are quite marked at the B cell level in b^4b^5 and b^5b^5 rabbits are also detected at the pre-B cell level. Also in progress are searches for kappa chain allotypes in pre-B cells of mutant rabbits of the BASILEA strain which are defective in expression of Ig with kappa type light chains.

In studies of the effects of allotype-suppression and its neutralization on the expression of b4 and b5 allotypes by B and pre-B cells from spleens and marrow of b^4b^5 rabbits, we found that pre-B cells of the suppressed allotype persist when B cells of that allotype are absent. The persistence of pre-B cells of the suppressed type supports the view that pre-B cells differ in their responsiveness to external influences such as anti-Ig compared to B lymphocytes. Surviving pre-B cells are a likely source of the B cells which appear within 24 hr of administration of "neutralizing" antigen to 14-23 day old suppressed heterozygotes as well as for B cells of suppressed allotype which appear during the recovery phase of allotype-suppression (Simons et al., 1979). We have found that cells bearing sIg of the suppressed type appear and increase toward normal proportions even when serum Ig levels of the suppressed type are markedly depressed (Simons, M., Ph.D. thesis, 1979 and Abdi, Scher and Mage, in progress).

Experiments are in progress to test the working hypothesis that Rheumatoid Factors produced by some individuals are reflections of exaggerated production of normal regulatory anti-globulins analogous to the regulatory idiotypic network described by Jerne. The specificity of such factors may not, however, be for idiotypes, but for allotypes or other characteristic antigenic determinants on immunoglobulins. We are using allotype-defined rabbits and human sera containing rheumatoid factors to investigate these questions. In rheumatoid patients, there clearly appear to be complexes of rheumatoid factors and circulating immunoglobulins of the patient. Isolated human rheumatoid factor does not seem to distinguish between specific rabbit C_H allotypes of IgG. Rheumatoid-factor-producing rabbits are now being generated to test the specificity of the analogous rheumatoid factors produced in the rabbit model (DeRito, Wilder, Karsh and Mage, in progress).

Antigen-specific surface receptors of B and T cells.

Rabbit and mouse splenic lymphocytes were radiolabeled by the lactoperoxidase technique, extracted with non-ionic detergent, immunoprecipitated with high-titered rabbit anti-kappa antisera and compared by SDS-PAGE. Mouse sIg peaks were reproducibly larger in size than rabbit sIg peaks (often greater than ten times). Neither differences in incorporation of label into the rabbit cell surface nor differences in average sIg density explain this result. We conclude that incorporated radioactivity may not reflect relative cell surface receptor density (Wilder et. al., 1979a). A rabbit cell surface Ig that bears light chain and V_H but lacks μ, γ- and α-allotypes was demonstrated. The molecule appears to be proteolytically labile but has an undegraded heavy chain that co-electrophoreses with μ chains on reduced SDS-PAGE. The similarities to published data on human and rat lymphocyte IgD strongly suggest but do not formally prove that we have identified the rabbit homologue of IgD (Wilder et al., 1979b). Staphylococcal protein A and several different immunoglobulins were radiolabeled to high specific activities (10^6 cpm/μg) by reductive methylation with tritiated sodium borohydride. The proteins retained excellent functional and antigenic properties. The reagents were used in a variety of assays for cell surface antigens as well as solution immunoassays. This radiolabeling procedure has become the method of choice for many cell surface and solution immunoassays currently being performed in the Laboratory of Immunology (Wilder et al., 1979c). T and B lymphocytes appear, at least partially to share idiotypic determinants. Sharing of V_H framework determinants is more controversial. In order to examine this question, we have prepared heterologous anti-rabbit and anti-mouse V_H antisera. Considerable characterization has been done of these reagents (Wilder et al., 1979d, and work in progress) and the sera are being used to further examine the T cell receptor question (Wilder et al., 1979e, and in progress). In addition we have supplied anti-rabbit V_H to a number of investigators for further testing immunochemically and in cell functional assays). We have studied the extent to which V_H framework determinants are represented on T cells in the rabbit using anti-a (V_H framework) allotype sera and anti-rabbit V_H sera made in goat (Wilder et al. 1979d,e) and chicken (work in progress). Flow microfluorometric analyses of splenic lymphocytes from hyperimmune rabbits demonstrated closely similar frequencies of cells stained with the anti-V_H framework antisera compared to anti-light chain antisera. To search for potentially hidden molecules, additional studies were performed with radioiodinated, detergent solubilized plasma membranes derived from immune spleen and lymph nodes. No evidence for a population of cells or a class of molecule(s) bearing V_H in the absence of light chain constant region determinants was obtained (Wilder et al., 1979e). One possible interpretation of our data is that only a small T cell subpopulation expresses V_H determinants. Conceivably, different immunization protocols might have expanded the proportion of such T cells. We are currently investigating other immunization protocols and have enriched for antigen binding T cell subpopulations to pursue this question.

Structure of immunoglobulins from allotype-defined rabbits.

The complete sequence of the variable region of a light chain from a rab-

bit anti-type III polysaccharide antibody of b4 allotype was determined. Comparisons with other sequenced rabbit kappa chains showed striking similarity to light chains with the rare amino terminal Ile-Val-Met sequence from antibodies elicited against Streptococcal A-variant and Micrococcus lysodeikticus carbohydrates. Residues 28-33 within the first complementarity-determining region (CDR) were identical in sequence to anti-A variant carbohydrate antibody light chains but the third CDR differed considerably. An unusual insertion of three amino acids following homology position 58 was found which has not been previously observed in rabbit kappa chains (Chersi et al., 1979a). The partial sequence (positions 29-71) of the variable region of light chains of predominately b5 allotype from the IgG of a single allotype-suppressed rabbit was obtained by traditional sequencing methods on isolated tryptic and chymotryptic peptides. The peptides from this region were isolated in relatively high yields and probably represent a dominant sequence. The framework sequence between positions 35 and 49 (FR2) is identical to an FR2 sequence commonly found in antibodies produced by b4 rabbits as well as in murine and human myeloma light chains, with the exception of an interchange of threonine for proline at position 43 or 44. This may be b5 allotype-related since to date all b4 light chains have had proline and a b9 light chain was found with arg at position 43. The fact that a dominant sequence could also be found for positions corresponding to the second CDR (50-56) in other species, confirms previous observations that this portion of the light chain is not extremely variable in the rabbit (Chersi et al., 1979b).

Breeding Experiments and Investigations of Chromosomal Organization.

To date, only one recombinant has been documented among 746 offspring of informative matings for the detection of V_H C_γ recombinant phenotypes. 490 of these progeny were informative for both hinge region (d11-d12) and C_H2 (e14-e15) allotypes. The analysis of the linked IgM and IgA allotypes in this recombinant as well as those of Kindt and Mandy and of Hamers et al. continues. A reinterpretation of the IgM phenotype of our recombinant was proposed based on reports of V_H-dependent conformational determinants of rabbit IgM (Mage, 1979). New data on the structure of Ig genes at the DNA level stimulated a re-evaluation of a number of questions concerning the arrangement and rearrangement of genes for rabbit Ig-heavy chains. A tabulation of the postulated heavy chain J-coding sequences from IgGs of known a-allotypes showed no apparent correlates of sequence and a-allotype for this portion of the V_H domain (Mage, 1979). Another general question raised was whether the apparent recombinations observed by ourselves and others within the heavy chain linkage group were intra-V_H, between V_H and J, intra-J, between J and C_H or indeed several of these. Some recombinations in the H-chain genetic region may be undetectable or poorly detectable because the recombinations result in disruption of mechanisms for efficient gene reorganization. Occasional, although infrequent, expression of certain genes after such recombinations might then lead to observation of "hidden or latent allotype or idiotype" production (Mage, 1979).

Significance to Biomedical Research and the Program of the Institute.

Immunogenetics continues to be at the forefront of our understanding of the nature of structural and regulatory genes in complex mammalian systems. As we increase our knowledge of the genetics and evolution of Ig genes and of the chemistry of the Ig proteins and of the DNAs which code for and regulate their production, the Ig system becomes one of the most valuable models for investigation and understanding of the functioning of complex genetic regions on chromosomes, and the molecular basis for cellular differentiation in higher organisms. The genetics, evolution and chemistry of cell surface isoantigens in the major histocompatibility locus are analogous in many respects to the Ig systems. Increased knowledge of Ig genetics has also provided models of direct relevance to the evolution and immunogenetics of cell surface antigenic systems. Understanding of the chemistry, genetics and evolution of Igs, the factors which control their genetic expression and the differentiation pathways of the cells which produce them is thus of broad fundamental interest.

Proposed Course of the Project.

Most of the projects described are in progress and continuing. Much of the evidence of linked inheritance and cis expression of V_H and C_H genetic information, allelic exclusion, imbalances in quantitative expression of "allelic" genes and "latent allotypes, or hidden genes" has been developed in the rabbit model. The phenomena continue to be studied intensively. In addition to our studies at the immunochemical, T cell, pre-B and B-cell levels, we are initiating experiments (Dr. L. Fitzmaurice, Research Expert), which will utilize cloned recombinant DNA molecules to investigate the above phenomena and obtain molecular descriptions of the events at the DNA and mRNA levels which contribute to these phenomena. The studies of specificity characteristics of Rheumatoid Factors in man and in animal models of Rheumatoid Disease will continue as a collaboration between this Laboratory and Drs. R. Wilder (Clinical Associate A & R/NIAMDD as of 7/1/79 and J. Karsh A & R/NIAMDD).

Appendix

Contract number N01 AI 82565-Maintenance and Breeding of Rabbits of Known Genotype and Their Use of Immunological Studies.

The objectives and methods are related to those outlined above. The contract has continued to provide outstanding support for production and maintenance of allotype-defined rabbits, immunizations, bleedings, and various short-term and long-term projects involving breeding, immunizations and suppression. Rabbits and reagents produced and supplied through this contract were made available to numerous other investigators at the NIH and were shipped to laboratories throughout the US and around the world.

Publications.

Ansari, A.A. and Mage, R.: Immunochemical Studies of the a Allotypes of Rabbit

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Chersi, A., Appella, E., Carta, S. and Mage, R.: The amino acid sequence of a variable region of rabbit b4 light chain from an anti-SIII antibody: comparison with light chains of the same subgroup from anti-A-variant carbohydrate antibodies. Molec. Immunol. In press, 1979a.

Mage, R.G.: A new look at the biological and genetic significance of rabbit heavy chain allotypes. Ann. Immunol. (Inst. Pasteur) 130C: 105-114, 1979.

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Simons, M.A., Hayward, A.R., Gathings, W.E., Lawton, A.R., Young-Cooper, G.O., Cooper, M.D. and Mage, R.G.: Expression of b4 and b5 kappa light chain allotypes by B and pre-B cells in allotype-suppressed and neutralized b^4b^5 rabbits. Eur. J. Immunol., in press, 1979.

Chersi, A., Mage, R.G. and Alexander, C.B.: Partial sequence of the variable region of IgG light chains of b5 allotype from b^4b^5 rabbit. Molec. Immunol., in press, 1979b.

Honors

Chairman, Editorial Board, Federation Proceedings
Editorial Board, J. Immunological Methods
Vice President D.C. Chapter, Sigma Xi
Invited Lecturer, International Colloquium, Institute Pasteur, Jan. 4-5, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AI 00037-12 LI

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Immunogenetics of Mouse Immunoglobulin and Genetic Control of Antibody Response

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Immunology

SECTION

INSTITUTE AND LOCATION National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This laboratory is mainly concerned with the genetic and cellular regulation of the expression of variable and constant region antigenic determinants present on light and heavy chains of immunoglobulin molecules before and after immunization with specific haptens. Idiotypes or V-region structural antigenic determinants associated with specific antibodies (e.g. anti polyfructosan, anti phosphorylcholine, anti α 1-6 dextran) have proven to be very useful as genetic markers or phenotypes in studies of genetic and cellular regulation of immunoglobulin expression. The preparation of monoclonal antibody (hybridomas) carrying specific idiotypes provide sources of large amounts of monospecific antisera that are invaluable in identification of specific markers in studies of interaction of cells in immunoglobulin expression.

PHS-6040
(Rev. 10-76)

A. Immune Response to Levan

I. Capacity to produce anti-inulin antibodies and cross reactive idiotypes appears late in ontongeny.

Adult BALB/c mice that have been immunized with bacteral levan (BL) produce anti-inulin and anti BL antibodies which exhibit cross-reactive idiotypes (IdX-G, IdX-A and IdX-B) found on inulin binding myeloma proteins. These mice also produce anti BL antibodies which do not bind inulin nor express IdX's. Young BALB/c mice immunized with BL or a variety of inulin conjugates do not produce anti-inulin antibodies but do produce anti BL antibodies. The late appearance of anti-inulin IdX^+ antibodies appears to be due to a late maturation of precursor cells and not to suppressor cells in the neonate. This has been shown by the fact that C.B20 mice that have received spleen cells from BALB/c neonates do not produce anti-inulin antibodies and that T cells from BALB/c neonates do not suppress the anti-inulin response of adult BALB/c. Furthermore, adult spleen cells transferred into neonates induce an anti-inulin response.

Although neonates do not produce IdX^+ anti-inulin antibody in response to immunization with levan, their serum contains $IdXA^+$ molecules which lack IdX-G and IdX-B and which do not bind inulin as BL. These $IdXA^+$ antibodies may possibly represent products of a "protoclone" which may subsequently develop into $IdX\text{-}G^+$. A^+ and B^+ anti-inulin antibody forming cells.

II. Suppression of antibody response by pretreatment with antibodies to cross-reactive idiotypes.

Pretreatment of BALB/c mice with antibodies directed at cross-reactive idiotypes of anti-inulin antibodies profoundly suppresses the inulin-antibody response to bacterial levan. This is shown by the failure of such pretreated mice to develop anti-inulin hemagglutinins or to develop antibodies detectable on isoelectric focusing. By contrast, B.C8 mice, which are congenic to C57BL mice but which possess the IgC_H^a haplotype fail to be suppressed by pretreatment with anti-idiotype antibody. This suggests that capacity to be suppressed by anti-idiotype pretreatment is determined by non-IgC_H genes. C57BL mice possess genes determining resistance to suppression and BALB/c mice possess genes determining sensitivity to suppression. As noted in Z01-AI-0030-11 LI, a similar situation pertains for the determination of clonal diversity and magnitude of anti-inulin response to BL immunization as assessed by isoelectric focusing. C57BL genes determined an increased response and a greater diversity. This is particularly striking since C57BL mice appear to lack the structural genes coding for the anti-inulin antibodies produced in response to BL. Efforts are in progress to determine whether the genes and those regulating the magnitude and diversity of the anti-inulin response are the same and to map these genes.

III. Anti bacterial Levan Monoclonal Antibodies.

Hybridomas were produced by fusion of spleen cells from BALB/c mice immunized with BL with the P31x63 cell line grown from a BALB/c plasmacytoma. P31x63 cell cultures produce and secrete IgG. Approximately 4000 clones were obtained and these were examined for anti BL and anti-inulin activity. 3/4000 clones showed anti BL antibodies and two of these also expressed idiotypes (IdX) that have been identified on normal BALB/c anti-inulin antibodies and also on myeloma proteins that bind inulin and BL. One of the hybridomas also expressed an idiotype A48 that has been found only on a myeloma protein that binds levan ($\beta 2 \rightarrow 6$) and not inulin ($\beta 2 \rightarrow 1$) polyfructosans. Monoclonal-anti BL antibodies from ascites fluid from BALB/c mice injected with these two hybridomas are being collected and purified.

B. Allotype Related Research.

I. α1-6 Dextran Response Linked to the IgCH Locus.

Responsiveness to α 1-6 dextran is independent of H-2 but is determined by the IgCH allotype of the strain. Mice of the $IgCH^b$ and $IgCH^j$ allotypes, such as the C57BL and CBA/J strains, are high responders while strains of other allotypes are low responders. Backcross analysis of low and high responders indicate that the gene controlling responsiveness is linked to the IgCH gene complex. F_1 hybrids of low and high responder strains indicate that responsiveness was inherited as a codominant trait.

II. Determination of the IgCH Domains that Express IgCH allotypes.

Cell lines of MPC11, an IgG2b plasmacytomas of BALB/c origin that had been treated with mutagens were found to have switched to producing IgG2a class of immunoglobulin identified by allotype analysis. Antisera prepared to the wild type identified MPC11 idiotype and IgG2b allotypes. Antisera prepared to the supernatant Ig secreted by the mutagen treated cell lines of MPC11 identified the MPC11 idiotype which cross reacted to MPC11 idiotype of wild type but also identified the IgG2a allotype. CH fragments were prepared from the MPC IgG2a protein by trypsin treatment of CH fragments H2L2 but lacking the CH3 domain, did not express IgG2a or IgG2b allotypes. Fc fragment consisting of the CH3 domain expressed the IgG2a but not the IgG2b allotype.

III. Studies of possible Linkage of λ_1 Allotype with λ_1^{hi} and λ_1^{lo} Regulatory and other Genetic Markers

Allotypic λ_1 markers, λ_1^{+1} and λ_1^{-1}, have been identified by alloantisera prepared against BALB/c λ_1 myeloma proteins in SJL mice. In addition, an antiserum has been prepared in a rabbit against the λ_1 Bence-Jones protein RPC 20. Using this heteroantiserum, it can be shown that some mouse strains have high λ_1 levels and others have low λ_1 levels. Genetic studies of crosses of strains with low levels of λ_1 with strains of high λ_1 levels show that the expression of level of λ_1 is controlled by regulatory

genes and the λ_1^{-1} is linked to the λ_1^{lo}. In addition we examined λ_1 allotype genes λ_1^{hi} in relation to other markers including IgCH Ly 1.1, Ly 2.1, Ly 5.1, $H\text{-}2^k$ C, ^{1}Hbb, and Gpi. No evidence of linkage of any of these markers to λ_1^{+1} or λ_1^{-1} has been found except for the ly 1.1 markers where in the appropriate progeny crosses, there was some evidence of loose linkage (75%).

IV. μ - δ Heavy Chain Recombinants.

44 backcross progeny of (C57BL/6 x DBA/2) x C57BL/6 were prepared for the genetic studies of the linkage of Lyb7.1 genes to the IgCH genes; these mice were also examined for serum and membrane δ allotype and for μ allotypes. Two progeny from the same litter were found to express serum and membrane δ allotype of the DBA/2 strain and μ, γ_{2a}, γ_{2b} and α allotypes of the C57BL. These two putative recombinants have now been proven to be true recombinants by appropriate mating tests.

C. Polymorphism of V-Region Markers in the Mouse

I. Phosphorylcholine antibodies of BALB/c and most mice of $IgCH^a$ haplotypes exhibit an idiotype designated T15 that is also present on a BALB/c phosphorylcholine binding myeloma proteins. Binding of antiidiotype antibody to the The T15 idiotype is not inhibitible by phosphorylcholine and, thus, is not in the combining site. Genetic studies of the T15 idiotype show that it is controlled by a gene linked to the IgC_H locus. Recently we prepared alloantisera to CBPC3, a phosphorylcholine binding myeloma protein from the C.B20 mouse which carried the IgA^{15} allotype marker of the $IgCH^b$ haplotype. Anti CBPC3 antisera produced in BALB/c, AL/N and A/He mice were made specific for CBPC3 idiotype by absorption with CBPC 105 (a CB20IgA myeloma protein) to remove anti-allotype antibodies. AL anti CBPC3 idiotype antibodies cross reacted with T15 myeloma protein. This cross reaction extended to anti-phosphorylcholine antibodies of normal C57BL and BALB/c. In addition absorption of this antisera with T15 removed the cross reacting idiotype (IdX) antibodies. Thereafter, the antisera was reactive with anti phosphorylcholine antibodies of C57BL but not BALB/c origin. BALB/c anti CBPC3 idiotype antibodies only reacted to CBPC3 myeloma protein and not with T15. This antibody combined with C57BL anti phosphorylcholine antibodies. These findings suggest polymorphism of V region markers coding for H chains of anti-phosphorylcholine antibodies. One marker is shared by BALB/c and C57BL mice while the other is present only in C57BL. The heavy chains of both the CBPC-3 and T15 myeloma proteins have been completely sequenced and their homology is very striking. They differ at positions 14, 16, 40 and 120. The latter is within the newly recognized J segment.

PUBLICATIONS:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AI-00038-07 LI

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Structure and Activity Studies on Immunologically Important Cells and Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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6.5	4	2.5

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Pa-2 stimulation of murine lymphocytes causes the production of soluble lymphocyte stimulating factors (LSF). These LSF are produced by splenic T cells and thymus cells. Fractionation of thymocytes by peanut agglutinin into non-agglutinable cells (about 10%) and agglutinable cells (90%) followed by removal of the PNA and stimulation with Pa-2, showed that the non-agglutinable thymocytes were responsible for the production of LSF. These helper factors have been separated into 6 major pools by affinity chromatography. While each stimulates DNA synthesis and immunoglobulin production by spleen cells, five are mitogenic for thymocytes or T cells and the sixth is B cell specific.

One of the earliest biochemical events following mitogenic lectin stimulation of lymphocytes is the activation of a membrane enzyme system which causes the methylation of phosphotidyl ethanolamine. A phosphocholine degrative pathway must also be activated in order for the cells to synthesize DNA or to produce soluble factors (LSF).

The fluorescence activated cell surface has been used to identify lectins which differentially bind to subsets of lymphocytes. Several such lectins have been identified and are being used to fractionate lymphocyte subsets.

I. Stimulation of Lymphocytes - Soluble Factors

We have previously shown that the T lymphocyte specific mitogen, Pa-2, stimulates soluble helper factor production by BALB/c thymocytes and splenic T cells. This mixture of lymphocyte stimulating factors (LSF, formerly called BSF) contained several components which varied in their biological activities. LSF has now been produced by several strains of mice and proven to stimulate lymphocytes across H-2 barriers.

Our recent experiments indicate that all of the LSF produced by BALB/c thymocytes comes from a small subset of these cells. By the use of differential agglutination with a lectin isolated from peanuts (PNA), murine thymus can be separated into two fractions; those that do not agglutinate (PNA^-, ca 10%) and those that are agglutinated (PNA^+, ca 90%). After removal of the PNA with specific inhibitors and recovery of single cells, both fractions were stimulated with Pa-2. Only the PNA - fraction produced LSF or synthesized DNA.

Preparations of LSF were known to contain several biologically active components. Affinity chromatography on columns of insolubilized Concanavalin A fractionates LSF into 6 active pools (I-VI), which appear to be glycoproteins. Pools I to V stimulate DNA synthesis and immunoglobulin production (measured by the reverse plaque technique) in spleen cells from normal mice but not from athymic nude mice. They also stimulate DNA synthesis in T cells and thymocytes. Furthermore, when tested on PNA fractionated thymocytes, the PHA^- fraction responded 100-200 fold better than the PNA^+ fraction.

These results indicate that the LSF in pools I-V, which appear to be some of the molecules involved in normal cellular control of immune responses, are produced by a small set of thymocytes contained in the PNA^- fraction and cause cell division only in thymocytes with same PNA^- fraction. The populations of cells in this fraction appear to largely overlap with cortisone resistant thymocytes.

On the other hand, the material in pool VI shows little or no mitogenic stimulation of thymocytes, but is the only LSF to stimulate DNA synthesis or increase the number of IgM secreting cells in the athymic nude mouse spleen. It is unclear whether this LSF acts directly on the B lymphocyte, or requires the presence of an auxillary cell.

We are presently investigating the biochemical properties of these LSF and attempting to further delineate the cells which produce them and their target cells.

II. Stimulation of Lymphocytes - Early Biochemical Events

Our studies in collaboration with LCM/NIMH have shown that Concanavalin A, a mitogen that stimulates DNA synthesis in T cells, induces a transient activation of membrane phospholipid methylation followed by an increase in degradation of methylated phosolipids. This results in a maximum incorporation of

^{3}H-methyl groups into membrane phosolipids at 10 minutes followed by a return to baseline by 40 minutes. The dose response curves for Con A on phospholipid methylation (measured at 10 minutes) and DNA synthesis (measured at 48 hr) were nearly identical.

To further correlate phospholipid methylation with later cell division, analyses were performed using lymphocytes from athymic nude mouse spleen, thymus, and splenic B and T cells separated by adherence to anti-immunoglobulin coated (Mage) plates. In each case, Con A activated the membrane phospholipid methylation system only with cells that also responded with DNA synthesis (T or thymus cells). Additional experiments using several mitogenic and non-mitogenic lectins showed a complete correlation between activation of the methylation enzymes and subsequent DNA synthesis.

Inhibition of phospholipid methylation with S-isobutyrylthio-3-diazadinosine was effective only if the inhibitor was added prior to Con A stimulation. After this initial step, the inhibitor could be washed away without loss of inhibition. Both the methylation (10 min) and DNA synthesis (48 hr) were equally inhibited. If the inhibitor was added at the same time as Con A and left for the duration of culture, no effect was seen either on phospholipid methylation or DNA synthesis.

It was further found that calcium influx into the cell was dependent upon the activation of phospholipid methylation. However, the degradation of methylated phospholipids did not occur in the absence of calcium. In summary, the membrane transmethylases are activated by the binding of mitogenic lectins to the cell surface, causing a transient increase in monomethyl ethanolamine, dimethylethanolamine, and phosphatidyl choline. The accumulation of monomethyl ethanolomine apparently causes a decrease in membrane microviscosity. At this point calcium appears to enter the cell and an increased breakdown of methylated phospholipids occurs. The products are arachodonic acid, which may be further metabolized into thromboxane and prostaglandins, and lysolecithin which may activate guanidylate cyclase.

The activation of the membrane transmethylases to synthesize methylated phospholipids, and the subsequent activation of degrative enzymes (probably phospholipase A2) appear to be integral events in the stimulation of mitosis by lectins. We are currently testing other lymphocyte activators, such as lymphokines, to determine whether these enzymes are in a common pathway of lymphocyte activation.

III. Lectin Fractionation of Lymphocytes

In collaboration with LMI/NIAID and I/NCI we have surveyed approximately 3 dozen lectins for preferential binding to different subsets of murine lymphocytes. The contribution of this laboratory has been the preparation and fluorescence labeling of lectins. Various lymphocyte populations were stained with the fluorescent lectins and analyzed by flow microfluorometry. We have identified several lectins which bind differentially to subsets of lymphocytes.

These lectins have nominal specificities for galactose/N-acetylgalactosamine, N-acetyl glucosamine, or sialic acids. Lectins with the same nominal specificity do not usually show the same cell staining patterns. The lectins we have identified will be used to develop techniques for the separation of lymphocyte subpopulations. Further experiments will characterize the cells in each subpopulation recognized.

IV. Microsequencing of Pituitary Glycoprotein Hormone Pre-α Subunits

The isolation of poly-A containing RNA from bovine and mouse pituitaries, from a murine pituitary thyrotropic tumor and their translation in a cell free wheat germ biosynthetic system containing ^{3}H-labeled amino acids was accomplished by the collaborating unit (CE/NIAMDD). The precursors of the α subunit (pre-α) of the glycoprotein hormones were isolated by immunoprecipitation and SDS-gel electrophoresis. This laboratory carried out microsequencing analyses of the radioactively labeled chains. Partial amino acid sequences were determined for the N-terminal 22 residues of these three pre-α subunits. The data indicate that both murine pituitary and pituitary tumor α subunits are synthesized with a "signal" prepiece and that this polypeptide is at least 22 residues in length. These pre-α subunits appear identical. The data also show that leucine is common to 4 positions (12,15,19 and 22) in murine and bovine pituitary pre-α as well as in human placental choriogonadotropin pre-α (other workers) subunits.

This extends the homology among α subunits from various species and organs into their prepieces, furthering the suggestion that the α subunits from pituitary and placenta are products of the same gene. There are no immediate plans to continue this project.

PUBLICATIONS:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AI-00040-05 LI

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Genetic Control of Immunocompetent Cell Interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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E. Heber-Katz, Staff Fellow, LI/NIAID
R. Burger, Guest Worker, LI/NIAID
O. Werdelin, Guest Worker, LI/NIAID
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COOPERATING UNITS (if any) Dr. Hinrich Bitter-Suermann, Dept. of Pathology, Georgetown University Medical School

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SECTION National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD

INSTITUTE AND LOCATION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long range goal of this laboratory is to determine the function of histocompatibility-linked immune response (Ir) genes in the regulation of the immune response and in the control of immunocompetent cell interactions. At present, the research effort is directed in two main areas: 1) the role of the I-region associated (Ia) antigens and Ir gene products in macrophage-T lymphocyte interaction, macrophage processing and presentation of antigen, and T lymphocyte antigen recognition. 2) Genetic, serological, physicochemical, and functional studies of the antigens of the guinea pig major histocompatibility complex (the GPLA complex).

PHS-6040
(Rev. 10-76)

I. Role of the I-region in the Regulation of Immunocompetent Cell Interactions

a. Macrophage Processing and Presentation of Antigen

We have postulated that the site of expression of the histocompatibility linked Ir genes may be at the level of the antigen presenting cell or macrophage rather than in the antigen-responsive T lymphocyte. Thus, macrophages from a non-responder animal would lack the I-region gene product necessary to process or present the relevant genetically controlled antigen. In order to test this hypothesis we have begun a detailed study of the biochemical basis of macrophage-lymphocyte interaction and are attempting to gain an understanding at the molecular level of macrophage processing and presentation of antigen. We have used as a model antigen the simple trinitrophenyl hapten (TNP) which can easily be covalently coupled to the macrophage surface. We initially examined the effects of anti-TNP antibody on the ability of TNP-modified macrophages to stimulate TNP-specific T cell proliferative responses in vitro. The addition of anti-TNP to TNP-modified macrophages immediately following conjugation inhibited their ability to stimulate TNP-specific T cell proliferation. However, anti-TNP had no effect on the TNP specific response to TNP-modified macrophages that had been cultured overnight before addition to primed T cells. This result initially suggested that macrophage presentation of the TNP determinant is not simply a surface display phenomenon and that the macrophage must process membrane conjugated TNP in a manner so that it is inaccessible to anti-TNP antibody to create the relevant immunogen recognized by T cells. However, recently we have been able to demonstrate that anti-TNP antibody can inhibit the proliferative response induced by cultured TNP modifield macrophages provided they have been freshly modified with the non-cross reactive dinitrophenyl (DNP) hapten. We could then study the effects of anti-hapten antibody on macrophages which simultaneously bore low concentrations of one hapten (aged) but high concentrations of the other (freshly modified). In these experiments we could demonstrate that the response to TNP-modified-aged-macrophages which were modified with DNP immediately prior to addition to culture was markedly inhibited with anti-TNP antibody. These experiments suggest that antigenic determinants are displayed on the macrophage surface, but under normal conditions a too low a density to allow efficient binding of anti-antigen antibody. At the present time we are conducting studies on the mechanism and requirements for anti-antigen induced inhibition of T cell proliferation . We are also extending this approach to examine the effects of antibody to soluble protein antigens on macrophage handling of such antigens. Hopefully, these studies will lend insights into the mechanisms whereby the products of the I-region play a role in macrophage presentation and processing of antigen.

We have previously demonstrated that treatment of macrophages with anti-Ia sera at any time following TNP conjugation resulted in a reduced ability of TNP-conjugated macrophages to stimulate a TNP-specific T cell proliferative response. These results suggested that anti-Ia sera interfere with the development of a TNP-specific immunogen on the macrophage and strongly suggest that an association exists between TNP-modified membrane proteins and Ia

antigens. We have therefore performed a detailed biochemical analysis of the relationship between the TNP moiety and the products of the major histocompatibility complex on the macrophage cell surface. Macrophages were TNP-modified, radio-iodinated, and lysed in detergent. When TNP-derivatized proteins were isolated using an anti-TNP immunoabsorbant and the presence of TNP-derivatized antigens in the eluted proteins determined by immunoprecipitation techniques no hapten modified Ia antigens were detected. Furthermore, when Ia antigens from TNP-modified cells were eluted from an anti-Ia immunoabsorbant, no proteins other than Ia antigens were detectable. It is thus highly unlikely that Ia antigens are strongly bound to a TNP-modified protein in a complex which withstands solubilization of the membrane. However, a complex maintained by the integrity of the intact cell membrane might very well exist.

The demonstration that Ia antigens are not derivatized on TNP-modified cells strongly suggests that the antigenic determinant recognized by guinea pig T cells does not consist of covalently trinitrophenylated Ia antigens. Further support for this data was obtained from studies where membrane preparations from TNP-modified macrophages were used in both primary and secondary cultures in place of TNP-modified macrophages. Surprisingly, membranes were effective stimulators of TNP-specific T cell proliferation in both primary and secondary cultures. However, effective stimulation was only observed in the presence of unmodified macrophages. A detailed study of the genetic requirements of this response demonstrated that the Ia antigens of the unmodified macrophages rather than the Ia antigens borne on the subcellular membrane fractions determine the histocompatibility restrictions on macrophage-T cell interaction. These studies effectively rule out the possibility that the major immunogen recognized by the T cell is the TNP determinant covalently coupled to macrophage Ia. Furthermore, these experiments suggest that Ia-positive macrophages can process and present antigens bound to membrane fragments.

b. Studies on the Physical Interaction Between Macrophages and T Cells.

Previous studies have suggested that physical contact between macrophages and T lymphocyte is an obligatory step in this cell-cell interaction. In order to examine the basis for this physical interaction between macrophage and T cell we have used monolayers of macrophages pulsed with soluble protein antigens as immunosorbents for T lymphocytes from guinea pigs primed to soluble protein antigens. When T lymphocytes were cultured for three successive 4 hour periods on such monolayers, they were selectively deprived of cells responding in an assay for antigen dependent proliferation against the antigen used for pulsing the absorbing monolayer, but maintained their response to other antigens; the lymphocytes adhering to the MΦ of the absorbing monolayer were enriched in responding cells. The proliferative response of F_1 T lymphocytes to antigen in association with MΦ of either parental strain could be absorbed leaving the response to antigen in association with MΦ of the other parental strain. The absorption of the proliferative response could not be inhibited by the addition of a large excess of soluble antigen to the medium of the absorption culture. These studies are in support of the view that T lymphocytes recognize and bind specifically to a complex of Ia antigen

and protein antigen at the surface of the MΦ.

c. Studies on the Genetic Regulation of the Autologous Mixed Leukocyte Reaction and on the Response to Aldehydes on Cell Surfaces.

It has been demonstrated that human T lymphocytes can be specifically sensitized to antigens present on autologous non-T cells. We have extended this experimental observation to the guinea pig system and demonstrated that guinea pig T cells will specifically proliferate when stimulated with syngeneic Ia positive macrophages. We have also shown that the syngeneic MLR exhibits both memory and specificity. Thus, T cells positively selected with syngeneic macrophages respond specifically in a second culture when stimulated with syngeneic rather than allogeneic macrophages. Two populations of F_1 T cells could be demonstrated, each responsive to unmodified macrophages of either parent. Furthermore, the response could then be specifically blocked by anti-Ia sera directed toward the stimulatory parental haplotype. The significance of these auto-reactive cells is still unclear. One intriguing possibility is that they represent a true reaction to self Ia antigens mediated by means of a low affinity receptor to self. Experiments are now in progress to test this hypothesis.

A second area of investigation related to the autologous MLR is the induction of T cell proliferation by macrophages which have been treated with neuraminidase and galactose oxidase (NAGO). In this experimental system, the macrophages may be either syngeneic or allogeneic to the responding T cell, but the proliferation can be specifically blocked by anti-Ia sera directed to the stimulatory macrophage. To further probe the genetic regulation of this response, we have examined whether 2 clones of F_1 T cells exist which respond specifically to aldehyde bearing macrophages of one parent, but not the other. Using the technique where proliferating cells can be selectively killed by incorporation of bromodeoxyuridine (BUdR) followed by exposure to light, we have shown that the F_1 T cells responding to modified macrophages of one parent are eliminated by exposure to aldehyde bearing macrophages of either parent. However, the stimulation produced by parental macrophages is still blocked by anti-Ia sera directed against the stimulatory parent. Thus, stimulation produced by NAGO treated macrophages is an I-region mediated event that is not genetically restricted. These studies suggest that the Ia molecule may subserve a function other than a role in antigen processing, or as a target for a part of the T cell receptor, but perhaps may function as a trigger or second signal in T cell activation.

II. Genetic, Serologic, Physicochemical, and Functional Studies of the Guinea Pig Major Histocompatibility Complex.

Although the major interest of the laboratory has shifted over the past two years to an analysis of the products of the MHC in the regulation of immunocompetent cell interactions, these functional studies have required a continued interest both in the serologic makeup of the GPLA complex and in the production of well characterized, potent antisera to GPLA antigens. Indeed, wherever possible we have attempted to correlate structural and physicochemi-

cal studies with functional studies. Several different areas have been studied during the past year:

a) Structural and Functional Studies of β_2-microglobulin - We have demonstrated two previously undescribed guinea pig molecules reactive with anti-guinea pig β_2-m. The first molecule was composed of a 36,000 dalton glycoprotein associated with β_2-m and was found on guinea pig thymocytes, but not lymphocytes. The second molecule was a 40,000 dalton glycoprotein associated with β_2-m and was found on both guinea pig thymocytes and lymphocytes. By structure, chemical composition, association with β_2-m, and tissue distribution, the first molecule is an attractive candidate for the guinea pig homologue of the murine thymus-leukemia (TL) antigen, whereas the second fits the criteria for the guinea pig homologue of the murine Qa-2 antigen.

We also examined the effects of a goat anti-guinea pig β_2-m serum on a number of T lymphocyte functions in vitro. Anti-β_2-m serum produced a marked inhibition of the response of peritoneal exudate T cells to antigen and mitogen stimulation. Surprisingly, a marked activation of lymph node T lymphocyte proliferation was observed in the absence of antigen or mitogen stimulation. The stimulatory effect of anti-β_2-m serum was specific for β_2-m and could not be blocked by antisera to the antigens of the guinea pig MHC. These studies suggest that β_2-m may play some critical role in the immune response at the level of T cell activation.

b) Role of C4 in the Cellular Immune Response - Previous studies have demonstrated that the structural gene for guinea pig C4 was linked to the GPLA complex. Because of the demonstration that antisera to human C4 inhibit the human mixed leukocyte reaction and the response to the mitogen, phytohemagglutinin, we undertook an intensive study of the possible role of C4 in the afferent phase of immune recognition. Antisera to C4 were raised in guinea pigs, rabbits and goats and tested for inhibition of the proliferative response to antigens, mitogens and alloantigens. In a large number of experiments no inhibition was found when the various cultures were formed in the presence of high titer antisera to guinea pig C4. Furthermore, lymphocytes from C4 deficient guinea pigs responded as well as inbred strain 13 lymphocytes to specific antigen or mitogen induced proliferation, indicating a normal capacity of T cells from C4-deficient animals for recognition of antigenic stimuli and for proliferation. Macrophages from C4D animals were able to effectively stimulate T cell proliferation, suggesting a normal capability for antigen or mitogen presentation. These data indicate that the demonstrated functional association of C4 and MHC-mediated reactions in the human does not generally apply to another species.

c) Antibodies to Guinea Pig Anti-Thrombin III Induce T Cell Proliferation - In the course of studies investigating the reaction of a goat anti-guinea pig immunoglobulin serum with guinea pig lymphocytes, it was noted that the serum reacted with both T and B cells as determined by indirect immunofluorescent staining and complement mediated cytotoxicity. In addition, the antiserum (or $F(ab')_2$ fragments) stimulated the proliferation of T lymphocytes, yet had no mitogenic effects on B lymphocytes. Absorption of

the antiserum with sepharose-bound IgG removed all detectable reactivity of the antiserum with B cell or serum Ig, but did not reduce its capacity to bind to or stimulate T cells. Immunoelectrophoretic studies revealed that, in addition to γ-globulins, the serum also recognized an α_2-globulin which was subsequently characterized as a 68,000 dalton glycoprotein that bore no identifiable Ig determinants. This protein was then purified by ion exchange chromatography and immunoabsorbent chromatography, and it was found that the cytotoxic and mitogenic activity of the antiserum could be abrogated by addition of the purified α_2-globulin. Functional studies and purification on heparin-sepharose affinity columns demonstrated that the α_2-globulin was anti-thrombin III. Studies to evaluate the importance of the cross reaction between this major serum protease inhibitor and an antigen on guinea pig T cells are in progress. The relevant antigenic determinant on the molecule was resistant to reduction and alkylation in 7m guanidine and to treatment with 2% SDS at 100°, yet was sensitive to periodate oxidation, suggesting it was carbohydrate in nature.

d) Production of Monoclonal Antibodies to Guinea Pig Cell Surface Antigens - In order to obtain more useful reagents for functional studies of the role of Ia antigens in immunocompetent cell interactions, spleen cells from BALB/c mice immunized to the Ia-positive L_2C leukemia of strain 2 guinea pigs were fused to the NS1 mouse myeloma. Culture supernatants reactive with guinea pig Ia antigens were obtained by screening against the Ia-positive leukemia cell line and its Ia-negative variant. Selected cultures were cloned and injected into pristane primed mice. Ascitic fluid from five independently derived hybrids appeared to detect Ia antigens in that they reacted with the Ia-positive leukemia cells, normal spleen cells, and to a lesser extent thymus, yet did not react with the Ia negative BZ-L_2C cell. Studies of the molecular characteristics of the antigens identified by these monoclonal antibodies are in progress. In addition, studies of the effect of these antisera on T cell and macrophage functions are also in progress. These data clearly demonstrate the usefulness of the hybridoma technique for the production of monoclonal antibodies to xenogeneic antigens if a sufficiently selective screening system is available.

e) Studies of the Induction of Transplantation Tolerance Following Allogeneic Spleen Transplantation - Previous studies in the rat by Dr. H. Bitter-Suermann have shown that immunocompetent rat spleen allografts could survive indefinitely in the absence of immunosuppression and that accepted spleen allografts induced a donor specific unresponsiveness for skin allografts. Because of our experience with in vitro assays of immunologic function in the guinea pig, we have collaborated with Dr. Bitter-Suermann to extend his experimental observations to the guinea pig. We have shown that strain 13 spleens can be transplanted to strain 2 guinea pigs and that in the absence of immunosuppression the splenic allograft is present up to 3 months following grafting. Preliminary studies suggest that recipients of spleen allografts are tolerant of skin derived from the donor strain. Further studies of the serologic makeup of the grafted spleen and the immunologic mechanisms involved in this unusual system of transplantation tolerance are in progress.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AI-00147-04 LI

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
The Mechanism of Activation of Thymus-Derived Lymphocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: Ronald H. Schwartz, M.D., Ph.D., Sr. Investigator, LI/NIAID

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LAB/BRANCH
Laboratory of Immunology

SECTION

INSTITUTE AND LOCATION National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.25	1.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project focuses on the function of immune response (Ir) genes in the activation of thymus-derived (T)-lymphocytes, using a secondary proliferation assay. By a slope analysis of cell dose-response curves, we have shown that three different cells are involved in the proliferative response, a nonimmune antigen-presenting cell, an antigen specific, primed T lymphocyte, and a non-immune, recruitable T lymphocyte. Experiments with radiation chimeras demonstrated that, of these three cell types, only the antigen-presenting cell has to possess high responder Ir genes. However, the proliferating, primed T lymphocyte must also have matured in a high responder environment. Studies of the proliferative response to the antigen, pigeon cytochrome c, demonstrated that two complementing Ir genes could control the response to a single antigenic determinant and that both gene products must be present in the same antigen-presenting cell in order to stimulate a response.

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(Rev. 10-76)

In our studies of the role of Ir gene products in T-lymphocyte immune responses, we have utilized the proliferative response of Peritoneal Exudate T-Lymphocyte-Enriched Subpopulations (PETLES), an assay developed in this laboratory several years ago. In addition we have recently set up a lymph node cell proliferation assay which also measures primed T lymphocyte function. During the past year we have demonstrated that 3 different cells are involved in the proliferative response, that immune response (Ir) genes need only be expressed in the antigen-presenting cell, and that the proliferative response to pigeon cytochrome c involves the recognition of a single antigenic determinant controlled by two MHC-linked Ir genes.

It has been known for many years that two cell types are involved in the proliferative response to soluble protein antigens. One of these cells is the antigen-presenting cell, a nonimmune, radioresistant, Ia-antigen bearing cell which lacks Ig and Thy 1 determinants. The most likely candidate for this cell type is one in the monocyte-macrophage lineage. The other known cell type is the antigen-specific primed T lymphocyte which is of the Ly 1^+2^- lineage. However, when we attempted to do log-log plots of cell dose-response curves, the slope for several different antigen-primed populations was always 3, suggesting that more than two cells were involved in the response. By adding different cell populations in excess over the primed population, we could convert the slope from 3 to 2, thus identifying the various cells. Both irradiated spleen cells and nylon wool-passed spleen cells from nonimmune mice could convert the slope from 3 to 2. Addition of both populations in excess reduced the slope from 3 to 1, suggesting that each population contained a different cell. Because the irradiated nonimmune spleen cells are known to possess antigen-presenting activity it was assumed that this population contained the macrophage. The nylon wool passed spleen cells, on the other hand, are depleted of macrophages and contain mostly nonimmune T lymphocytes. Because its filling capacity was eliminated by irradiation, we concluded that this newly identified cell type was probably a recruitable (i.e. non-antigen specific) T lymphocyte which divided in response to mitogenic signals from the stimulated antigen-specific T lymphocyte.

Our previous studies on the localization of immune response genes in the responding PETLES population had demonstrated a requirement for the antigen-presenting cell to come from a high responder strain. In pursuing these studies further, we have now demonstrated that the T-lymphocyte does not have to be from a genotypic high responder provided the cells mature in the environment of a high responder. This conclusion was reached from studies with radiation chimeras. T-cell depleted bone marrow cells from low responder mice were injected into lethally irradiated high responder F_1 mice and allowed to develop into mature T cells. Such chimeras do not respond to the antigen, suggesting the requirement for at least one high responder cell type. Because we knew that the antigen-presenting cell was one such cell type, we had to provide this cell in order to assess the requirements for the T-lymphocyte. This was accomplished by transferring chimeric spleen cells to an acutely irradiated F_1 along with T-cell-depleted F_1 bone marrow. These animals now responded to the antigen.

Thus, only high responder antigen-presenting cells were required. However, the environment in which the T cells develop was also critical. When chimeras of the type [high responder F_1 bone marrow into lethally irradiated low responder hosts] were created, they failed to respond to the antigen even though they possessed high responder antigen-presenting cells derived from the bone marrow. Thus, the T cell must learn to recognize the appropriate Ir alleles from radioresistant cells of the host when they differentiate from marrow stem cells. Once a high responder repertoire is fixed, the T cells can then recognize antigen when it is presented on a high responder macrophage.

In deciphering the mechanism of action of Ir gene products, it was deemed necessary to investigate the types of antigenic determinants recognized by T lymphocytes to ascertain whether the repertoire of receptors on these cells was any different from that of B lymphocytes. To facilitate such experiments we turned to the small globular proteins as antigens, because they provided us with probes for which we possessed a thorough understanding of the amino acid sequence and a precise knowledge of the three-dimensional structure. Thus, we could localize the antigenic determinants by cross-stimulation with species variants and enzymatic or chemical cleavage fragments of the molecules. For the last few years we have focused on the T-lymphocyte proliferative response of B10.A mice to pigeon cytochrome c. This response appears to be directed at a single determinant comprised of three amino acid residues from different parts of the molecule: the isoleucine at position 3, the glutamine at position 100 and the lysine at position 104. These three residues lie next to each other on the outer surface of the back face of the molecule. Although these results suggest that T cells recognize three dimensional determinants, cyanogen bromide cleavage fragments of the molecule could stimulate the T cells primed to the whole molecule. In fact, one fragment, residues 81-104, containing only two of the three critical amino acids and lacking any of the α helical content this segment possesses in the native structure, stimulated better than the whole molecule on a molar basis. Furthermore, this fragment was immunogenic and the response was under the control of two MHC-linked Ir genes, just as was the response to the whole protein. These results have raised the possibility that T-cell receptors recognize antigen fragments on the surface of macrophages rather than intact molecules. Mixing experiments demonstrated that many of the responding T cell clones were recognizing the two critical amino acids found in the fragment as part of the same determinant. This result demonstrated for the first time that two Ir genes could control the immune response to a single antigenic determinant. Finally, antigen-presentation experiments showed that both Ir gene products must be expressed in the same presenting cell in order to generate an immune response to pigeon cytochrome c.

Significance to Biomedical Research

Our attempts to understand the mechanism of T-lymphocyte activation are of fundamental importance to a basic understanding of how the immune

system functions. Since this system plays a critical role in the bodies defense mechanisms against infectious diseases, tumors and transplanted tissues, achieving our goal would hopefully provide major insights into how to manipulate the immune system for the benefit of the patient.

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I N D E X

LABORATORY OF INFECTIOUS DISEASES

SUMMARY STATEMENT -- Annual Report of the

Laboratory of Infectious Diseases
National Institute of Allergy and Infectious Diseases
October 1, 1978 to September 30, 1979

The main focus of the research programs of LID continues to be the natural history and prevention of the three major uncontrolled, acute infectious diseases of man--acute respiratory tract disease, viral hepatitis and acute gastroenteritis. Broad advances were made in each of these areas during 1978-79.

Hepatitis

Hepatitis B virus (HBV) - Although a hepatitis B vaccine prepared from HBsAg (surface antigen) purified from the plasma of chronic carriers of the antigen is feasible and probably cost-effective for its contemplated uses in the United States, such a vaccine probably will not be cost-effective for the people most in need of a vaccine in the developing world. Therefore, additional studies on alternative methods of purification and inactivation, comparisons of monovalent vs. bivalent or polyvalent vaccines, studies of different combining ratios of monovalent vaccines into bivalent preparations, different vaccine dosages, different vaccination schedules, different routes of administration, studies of possible adjuvant and their interaction with vaccine preparations and further characterization of the immunizing antigens themselves are underway. Four modified vaccines have been prepared. Two of these have been extracted with ether-tween 80; 2 are alum-precipitated. Preliminary results from studies in volunteers indicate that certain of the vaccines are much more immunogenic than any previous hepatitis B vaccine tested. Thus, they produce antibody to hepatitis B surface antigen in two to three weeks instead of two to three months (Purcell, McAuliffe).

Hepatitis A virus (HAV)- Only one antigen, hepatitis A antigen (HAAg) has been associated with type A hepatitis infections to date. An immunofluorescence assay was developed for hepatitis A antigen that has permitted us to study the tissue distribution of viral infection in chimpanzees and marmosets. HAAg was detected in the liver, germinal centers of lymph nodes and spleen, and basement membrane of the kidney. The latter sites probably represent sequestration of antigen released from the liver into the circulation. Interestingly, viral antigen was not detected in the intestines. Thus, we have not been able to demonstrate an enteric phase of multiplication for the hepatitis A virus.

The development of an immunofluorescence test for hepatitis A viral antigen permitted Provost and Hilleman to identify hepatitis A virus multiplication in tissue culture. The inoculum for their tissue culture studies was a strain of HAV that had been passaged in marmosets for 31 times. Multiplication of hepatitis A virus in tissue culture after marmoset passage was confirmed

in LID. Three strains of HAV have been serially passaged in marmosets; at least two of these (and possibly the third) produce hepatitis A viral antigen detectable by immunofluorescence in African green monkey cells. Serial passage in this cell line is currently being attempted, and the parameters that affect growth in culture (number of passages in marmosets, titer, etc.) are being examined.

As with type B hepatitis, pathologic mechanisms involved in type A hepatitis are not understood. It appears that type A hepatitis rarely if ever progresses to chronic disease. However, this assessment is based upon a limited serologic analysis of chronic hepatitis patients. It is now possible to directly examine liver biopsies from patients with chronic non-B hepatitis to determine if their hepatocytes contain HAAg using the immunofluorescence technique developed in LID. Such studies have confirmed that HAV is not an important cause of chronic hepatitis (Feinstone, Purcell).

Non-A, non-B, hepatitis viruses - One approach to the identification of non-A non-B hepatitis-associated antigens is the application of techniques useful in the detection and identification of HBV and HAV: immune electron microscopy, radioimmunoassays and immunofluorescence. By application of radioimmunoassay techniques, a serum antigen associated with two cases of well-characterized non-A, non-B hepatitis was identified. The appearance and disappearance of this antigen in the serum was temporally related to hepatitis in these two individuals, and the antigen was shown to be particulate and biophysically characterizable by ultracentrifugation procedures. Preliminary antibody surveys suggested that antibody to this antigen was widespread among humans and chimpanzees and that, if it is an antigen associated with non-A, non-B hepatitis, it is not associated with the major cause of this disease (Purcell, Feinstone).

A number of unsuccessful attempts to transmit non-A, non-B agents to primates were carried out in the past. However, recently two successful transmission studies were completed simultaneously, one by the Bureau of Biologics and the other by the Clinical Center Blood Bank in collaboration with our laboratory. In both studies the incubation period in chimpanzees was comparable to that for non-A non-B hepatitis in man, and both biochemical and histological evidence of hepatitis was obtained. In our collaborative study, plasma or serum from patients with chronic non-A non-B hepatitis as well as from patients with acute infections transmitted disease to chimpanzees. Thus, proof of chronic carriage of non-A non-B viral hepatitis agents has been obtained (Feinstone, Purcell).

As part of our study of non-A, non-B hepatitis in chimpanzees, serial liver biopsies have been obtained. These biopsies were studied intensively by thin section electron microscopy and morphologic evidence for two types of non-A, non-B hepatitis was observed. One produces characteristic cytoplasmic changes that consist of proliferation, thickening and duplication of endoplasmic reticulum. The structures so formed appear to be cylinders of modified membrane that enclose a tube of rough endoplasmic reticulum. Although the

structures do not resemble known viruses, they do "breed true", that is, the same types of structures are seen in liver biopsies of chimpanzees that have received the same inoculum or that have participated in serial transmission of the agent. In contrast, the other non-A, non-B agent produces predominantly nuclear changes that consist of shrinking, heterogeneous staining of the chromatin and, in some cells, clusters of intranuclear virus-like particles. These changes also "breed true". The nature of the structure is not well understood. Furthermore, the relationship to the etiologic agents may not be a direct one. Nevertheless, they provide the first means for differentiating between non-A, non-B agents (Y. Shimizu, Feinstone, Purcell).

Acute Gastroenteritis

Important advances were made last year in our understanding of the etiological agents of acute infectious nonbacterial gastroenteritis, a syndrome that affects a broad segment of the population. Equally impressive advances were made toward our goal of preventing such disease by immunoprophylaxis.

Cultivation of type 2 human rotavirus in vitro - Rotaviruses are now recognized as the major cause of serious diarrheal disease of early life. They are responsible for significant morbidity in the USA and undoubtedly cause a major share of the many millions of fatal diarrheal illnesses that occur in developing countries each year. Although laboratories throughout the world have sought to grow these viruses serially and to high titer in tissue culture all attempts until now have failed. Several years ago we succeeded in passaging a type 2 human rotavirus strain 24 times in human embryonic kidney but only limited viral replication occurred. During the past year, the long sought after goal of growing human rotavirus to high titer in culture was achieved.

Initially, 42 stool specimens known to be positive for rotavirus by electron microscopy (EM) were tested for their ability to infect primary African green monkey kidney (AGMK) cell cultures. Only 5 specimens induced fluorescent antibody stainable antigens in 10% or more of cells. In order to amplify virus that was efficient in initiating infection in culture and hence increase the probability of the emergence of a cell culture adapted mutant, a human type 2 rotavirus (strain "Wa") was administered orally to gnotobiotic piglets. These animals developed mild, transient diarrhea. Virus was passaged subsequently 10 times in gnotobiotic piglets in collaboration with Dr. E.H. Bohl, Ohio Agricultural Research and Development Center, Wooster, Ohio. The development of diarrhea was variable but piglets at each passage were infected as indicated by the detection of a large quantity of viral antigens in intestinal contents.

Attempts were made to initiate serial passage of the "Wa" strain in AGMK cells using a stool filtrate derived from patient "Wa" and intestinal aspirates collected at the first, second, third, and eleventh passage in piglets. A standardized technique was used, but serial passage in culture was achieved only with the eleventh passage material from piglets. Prior to inoculation of AGMK cells, virus suspension was incubated with trypsin

at a final concentration of 10 ug/ml for 1 hour at 38°C since this procedure had been shown to enhance the infectivity of some animal rotaviruses for tissue culture. Inoculated AGMK cell cultures were centrifuged at low speed, a procedure that also increases the infectivity of some animal rotaviruses. During 14 serial passages in AGMK cells the "Wa" strain attained a titer of $10^{3.0}$ to $10^{6.6}$ fluorescent focus (FF) units per ml.

Two approaches were used to establish the identity of the AGMK-adapted virus as a human rotavirus. When studied by polyacrylamide gel electrophoresis the viral RNA pattern of the tissue culture adapted strain was identical to a previously recognized human rotavirus RNA pattern. It differed, however, from the patterns of six animal rotaviruses that grow readily in cell culture. Bovine, simian and porcine tissue culture adapted rotaviruses were also distinct from the culture-adapted "Wa" strain when tested by neutralization in cell culture. Thus, the tissue-grown "Wa" strain appears to be type 2 human rotavirus and not a tissue culture-adapted animal rotavirus contaminant acquired in the laboratory. Cultivation of type 2 human rotavirus in tissue culture should facilitate a more detailed examination of its properties and permit us to manipulate its genome with the intent of developing attenuated mutants for use in prevention of serious diarrheal disease of early life (Wyatt, Kalica, Kapikian, Chanock).

Development of ts mutants of calf rotavirus - As part of our effort to modify the virulence of rotavirus, 8 ts mutants of the UK strain of calf rotavirus were developed using 5 azacytidine or nitrosoguanidine as mutagen. The 8 mutants had a shutoff temperature for plaque formation of 38°C or 39°C and appeared to be relatively stable genetically. It is our intention to use these and subsequent mutants to probe the function of individual genes of the rotavirus genome. ts mutants will also be used as donors of ts genes that will be transferred to the tissue culture-adapted human rotavirus via genetic reassortment in an attempt to attenuate the human virus. Bovine rotavirus genes will probably prove to be host restricted in human cells, i.e., hr, and this may offer another mechanism by which genes transferred from the bovine virus could bring about attenuation of the human virus (Greenberg).

Possible use of animal rotavirus to vaccinate against human rotaviral disease - Last year we reported that in utero infection of calves with a bovine rotavirus induced resistance to challenge at birth with type 2 human rotavirus. This stratagem was necessary since calves are susceptible to the disease producing effects of human rotavirus only during the first few days of life. Hence the need to immunize in utero in order to be able to challenge with the human virus shortly after birth. Completion of this study during the past year indicated that the bovine rotavirus was sufficiently related to the human virus to be considered as an immunizing agent against human rotaviral disease. Safety testing of a suspension of bovine rotavirus is currently being completed. No adventitious agents have been detected. Since this rotavirus was recovered from a domestic animal in the UK, additional safety tests are being performed at Plum Island Animal Disease Center, USDA, in collaboration with Dr. C.A. Mebus. Disease produced in calves by

the UK strain was similar to that seen with the U.S. bovine rotavirus. However, the UK isolate induced disease in gnotobiotic piglets, while the U.S. isolate did not. When testing is complete, USDA will be asked for permission to release this strain for distribution and study in man (Wyatt).

Norwalk and related 27 nm viruses - A radioimmunoassay blocking (RIABL) test for detection of antibody to the Norwalk agent was employed to continue our survey of the importance of this virus in epidemic gastroenteritis. Sixty-five outbreaks were studied serologically and 20 (31%) appeared to be associated with the Norwalk virus as indicated by seroresponse of affected individuals. This information reaffirms our previous conclusion that the Norwalk virus is a major cause of epidemic gastroenteritis. The new Norwalk-associated outbreaks occurred in a wide variety of situations including grade schools, summer camps, nursing homes, and cruise ships. In two Norwalk-associated outbreaks, originally studied by the CDC, water borne transmission appeared to be the mode of spread. Interestingly, workers in Australia associated Norwalk virus etiologically with several very large oyster borne outbreaks of acute gastroenteritis; this was confirmed in our laboratory (Greenberg, Kapikian).

Antibody prevalence to the Norwalk virus - A large battery of age stratified sera from adults from various parts of the world was studied for Norwalk antibody. Prevalence rates were stable and similar in adults from the U.S., Switzerland, Belgium, Yugoslavia, Ecuador, Bangladesh, and Nepal (approximately 70%). On the other hand, children from the U.S. and Yugoslavia acquired antibody far more slowly than did children in Ecuador and Bangladesh. In Ecuador and Bangladesh an antibody prevalence of greater than 70% was found in children 0-5 years of age. This high prevalence in early childhood suggests that Norwalk virus may play an important role in childhood gastroenteritis in underdeveloped countries. The low prevalence of antibody in young children in the U.S. is in keeping with our failure to incriminate this agent as a significant cause of infantile gastroenteritis (Greenberg).

Bacterial gastroenteritis - The solid-phase microtiter radioimmunoassay for the detection of E. coli heat labile enterotoxin was modified to a blocking test to detect antibodies to this enterotoxin. A comparison of the efficiency of the RIA blocking and the adrenal cell neutralization tests for detecting serological responses in individuals experimentally infected with toxigenic E. coli revealed that both techniques were efficient at detecting a seroresponse in ill volunteers. Nine of 10 volunteers who became ill developed an RIA blocking antibody response, and 7 had a serological response when tested by the adrenal cell method. Proportionately fewer non-ill volunteers responded when tested by either method. RIA blocking antibody titers were between 5- and 10-fold higher than adrenal cell titers, and preexisting antibody was detected somewhat more commonly by the RIA blocking method. When paired sera from adults with naturally occurring diarrhea in Kenya and Bangladesh were studied it was found that both methods detected a significant rise in antibody in approximately one-half of the patients shown to be shedding toxigenic E. coli. The age prevalence of serum anti-LT was investigated by

screening random sera obtained at Children's Hospital, Washington, D.C. and Junior Village. By 48 months of age, over 50% of the population studied had developed antibody to E. coli LT. In addition, it was found that over 70% of a group of University of Maryland students aged 17 through 26 had detectable serum anti-LT (Greenberg, Kapikian).

Influenza Viruses

ts Mutants for Immunization - One of the goals of the LID influenza virus program is to develop a safe, effective procedure for the prevention of influenzal disease. Although inactivated influenza vaccines, as now formulated, are effective they do not provide complete protection nor do they appear to retain their effectiveness when administered annually. For this reason we have attempted to develop a live attenuated vaccine that would mimic natural infection in its greater, broader and more durable protective effect. We sought to produce mutant influenza viral genes which would confer a satisfactory level of attenuation upon any recombinant virus into which they were transferred by genetic reassortment. The explosion of new information concerning the structure and function of the influenza virus genome provides a basis for deliberate manipulation of its genes for this purpose. Influenza viruses bearing specific, identifiable, attenuating mutations represent the vaccine strains of the future since the genetic basis for attenuation can be monitored directly during all phases of vaccine development, manufacture and utilization in man.

Our approach has been to develop ts mutations on genes that code for the non-surface proteins of the virus so that the mutant genes could be transferred into recombinant viruses bearing the surface antigens of any new epidemic or pandemic influenza virus. It is implicit in this approach that ts mutations are primarily responsible for the attenuation of recombinant viruses bearing ts genes. During the past year convincing evidence was obtained that this is indeed the case. When a series of ts-1[E] and ts-1A2 recombinants, previously evaluated in volunteers, was genotyped (i.e., the parental origin of each gene in the recombinant was determined by polyacrylamide gel electrophoresis) it was shown that only the ts genes from the ts donor parent segregated with the property of attenuation. This finding confirmed previous observations made in hamsters which indicated that restriction of growth of ts recombinants in the respiratory tract was a function of their ts genes (Markoff, Massicot, Murphy, Chanock).

Initially a master strain containing the ts-1[E] mutations was used as a donor of ts genes. These ts mutations were located on the genes coding for the P3 and NP proteins, the former involved in cRNA synthesis and the latter in vRNA synthesis. Recombinants bearing both of these ts genes were satisfactorily attenuated,immunogenic and genetically stable when tested in adults who had detectable neuraminidase immunity but who lacked serum antibody for the hemagglutinin antigen. However, ts-1[E] recombinants produced mild febrile reactions when given to individuals who lacked immunity to both viral surface antigens, i.e., hemagglutinin and neuraminidase. Also genetically altered (ts^+) virus was recovered from some doubly seronegative volunteers infected with a ts-1[E] recombinant.

When immunity to both surface antigens is absent only the degree of defectiveness of the vaccine virus determines attenuation and in this situation defectiveness must be greater than that produced by the ts-1[E] mutations. For this reason we developed the ts-1A2 set of mutant genes that specified a greater degree of defectivenss than the ts-1[E] mutations. The A/Udorn/72 ts-1A2 donor virus and its recombinant derivatives were more temperature sensitive, more stable genetically, and more restricted in growth in the respiratory tract of hamsters than the ts-1[E] parent or its recombinants. Further, unlike the ts-1[E] recombinants, the ts-1A2 parent and its recombinants were completely stable genetically during growth in hamsters. The ts-1A2 mutations were shown during the past year to be located on the genes coding for the P3 and P1 proteins, both of which are involved in cRNA synthesis, the earliest phase of viral replication (Massicot, Murphy, Markoff).

To date the ts-1A2 mutations have been transferred to recombinants bearing the surface antigens of A/Vic/75 (H3N2), A/Alaska/77 (H3N2) or A/HK/77 (H1N1) virus. Transfer of the two ts genes from the A/Udorn/72 ts-1A2 parent to a total of 11 such recombinants yielded viruses with a uniform set of properties: (a) 37°C shutoff temperature for plaque formation, (b) 10,000-fold suppression of growth of virus in the lungs of the hamster, (c) 100-fold restriction of viral growth in the hamster's nasal turbinates and (d) genetic stability after replication in hamsters (Murphy).

Based on these observations representative A/Vic/75, A/Alaska/77 and A/HK/77 recombinants were evaluated in adult volunteers, most (A/Alaska/77) or all (A/Vic/75 and A/HK/77) of whom lacked detectable immunity to both surface antigens. These recombinants were satisfactorily attenuated in each instance. The A/Vic/75 and A/Alaska/77 recombinants were effective in stimulating serum hemagglutination-inhibiting (HI) antibodies, but the A/HK/77 recombinant appeared to be poorly antigenic when measured by this criterion. However, when tested by the more sensitive, immunoglobulin-specific influenza A virus ELISA it was found that most of the A/HK/77 volunteers had developed a serologic response. In every instance virus recovered from infected adult volunteers retained the ts phenotype.

The A/Vic/75 ts-1A2 recombinant was satisfactorily attenuated, antigenic and genetically stable when tested in doubly seronegative children by Drs. Peter Wright and David Karzon (Vanderbilt). In contrast the A/Alaska/77 ts-1A2 recombinant, although it did not cause illness, was unstable genetically when evaluated in a doubly seronegative child by Drs. H.W. Kim and Robert Parrott (Children's Hospital, D.C.). Late in the course of infection this child shed virus that produced plaques in culture at 39°C but not at 40°C. Hence this virus was less ts than its A/Alaska/77 ts-1A2 parent, but more ts than A/Alaska/77 wild type virus (shutoff temperature of greater than 40°C). These observations highlight one of the central dilemmas inherent in the ts mutant approach. The same may be said for cold-adapted (ca) recombinants which on occasion have lost (a) the ca phenotype during infection of volunteers and (b) the ts phenotype during experimental infection of hamsters (Murphy, Markoff).

The mechanism for genetic alteration leading to loss of the ts phenotype was partially elucidated during the past year. Among the reoviruses, which have a segmented RNA genome, loss of the ts phenotype is usually the result of extragenic suppressor mutation (Ramig and Fields, 1979). This can be shown by backcrossing the ts^+ "revertant" virus with wild type virus and recovering ts progeny bearing the original ts mutation. A similar analysis was performed with the Alaska/77-ts-1A2 ts^+ isolate by mating it with Alaska/77 wild type virus. Twenty-two percent of progeny virus from this mating was ts and each ts clone possessed the P3 ts lesion present in the ts-1A2 parent. However, the ts segregants possessing the P3 ts gene were less temperature sensitive than P3 ts segregants derived from mating Udorn/72-ts-1A2 with wild type virus. This indicates that two types of genetic alteration occurred in the Alaska/77-ts-1A2 virus during replication in a seronegative child. One type involved a suppressor mutation in another gene (i.e., not the P3 gene) that partially corrected the ts phenotype of the ts-1A2 virus. The second type was an alteration affecting the P3 and P1 genes; this type of change could result from reversion of one of several distinct mutations or it could represent intragenic suppression. A mutation probably occurred on the P3 gene of the Alaska/77-ts-1A2 ts^+ isolate because this segment migrated more slowly on polyacrylamide gel than the corresponding RNA segment of the parent Alaska/77-ts-1A2 recombinant. This observation favors the possibility that intragenic suppression was responsible for decreased temperature sensitivity of the P3 ts segregants derived from the ts^+ isolate (Murphy).

The cumulative experience indicates that acquisition of the two ts-1A2 genes by H3N2 or H1N1 subtype virus was regularly associated with a level of attenuation satisfactory for individuals lacking antibody to both surface antigens of the virus. However, the experience with the A/Alaska/77 ts-1A2 recombinant indicated that genetic stability remains a formidable problem that must be resolved. It may be unrealistic to insist that live attenuated vaccine viruses possess ts, ca or host restriction (hr) mutations that are completely stable. The high mutation frequency of single stranded RNA viruses may make this a difficult, if impossible goal to achieve. For example, the spontaneous frequency of ts mutation for influenza A and VSV viruses has been observed to range from 1-2%. Perhaps we should focus our attention upon the consequences of genetic alteration that occurs in attenuated mutants during replication in vivo and determine whether such alteration results in increased virulence and whether altered vaccine virus poses a threat to contacts of vaccinees. Alteration or loss of laboratory markers of attenuation, i.e., the ts or ca phenotype, may not necessarily disqualify vaccine viruses that undergo such modification, especially if it results from "suppression" rather than true reversion. If complete genetic stability were an absolute requirement for vaccine virus, live poliovirus vaccine would not be available for the control of poliomyelitis. The attenuated poliovirus vaccine strains frequently undergo a modification in ts and other laboratory markers of attenuation during viral replication in man without causing disease in vaccinees except on very rare occasion. The spectrum of change in markers seen in polioviruses recovered from vaccinees suggests that "suppression" probably plays a major role in these modifications.

Cold-adapted (ca) Recombinants for Immunization - Two ca recombinants developed by Maassab (Univ. Michigan) were evaluated in doubly seronegative adult volunteers. These recombinants both possessed the maximum number of transferrable ca parental genes (i.e., P1, P2, P3, NP, M and NS) yet the A/Alaska/77 cold recombinant (CR-29) had a 39°C shutoff temperature, while A/HK/77 CR35 was more temperature sensitive (37°C shutoff). Nonetheless both CR-29 and CR-35 were satisfactorily attenuated and antigenic in adults. Furthermore, these recombinants retained the ts phenotype during replication in volunteers although some isolates of the CR-35 exhibited some genetic drift (37°C shutoff --> 39°C shutoff temperature) (Murphy).

Characterization of New ts Recombinants - Genetic studies of 136 new ts mutants of A/Udorn/72 virus were completed this year. Several unusual phenomena were observed during the characterization of these mutants. First, 56 of the ts mutants exhibited host dependent temperature sensitivity, i.e., they were ts when tested in one host cell but not ts when evaluated in another host cell system. This type of host dependent mutation was widely distributed throughout the viral genome and did not exhibit a predelection for a particular gene. Second, intracistronic complementation, without recombination, occurred with ts mutations that affected the genes coding for the P1, P2, NP and NS proteins.

The 136 mutants were assigned to 8 distinct, non-overlapping complementation recombination groups. This number of groups is in agreement with the number of influenza A virus RNA gene segments. This large suite of highly characterized ts mutants, 83 of which are single mutants, should prove useful in future studies of gene function and expression particularly in experiments in which rescue of cloned influenza DNA sequences from eukaryotic cells will be attempted (K. Shimizu).

Use of Recombinant DNA Techniques to Study Influenza A Virus - In view of the medical importance of influenza viruses there is a need to broaden our knowledge of the genetics of these viruses in order to design ways to more effectively prevent pandemics and epidemics. One approach is to use recombinant DNA technology that has been well-developed and has proven valuable in elucidating gene organization and expression in other appropriate host systems. Our general plan for this approach includes two phases. The first phase involves cloning each of the eight influenza gene segments using the E. coli K12-plasmid system (approved MUA #91). The second phase envisions the use of purified clones of influenza DNA sequences to examine viral gene expression in animal cell culture and to produce viral RNA (vRNA) in eukaryotic cells. To carry out this second phase we propose to utilize defective SV40 (lacking the late region of the genome that codes for capsid proteins) for construction of influenza-SV40 hybrid DNA molecules (submitted MUA #110). Introduction of the appropriate influenza-SV40 hybrid DNA molecules into eukaryotic cells may lead to transcription of cRNA or replication of vRNA from influenza viral DNA depending upon its orientation of insertion. Our goal is to devise procedures which would permit conversion of influenza DNA back to an influenza RNA negative strand

(i.e., vRNA) and eventually transfer such RNA into an influenza virus. In this manner, stable, site-specific mutations, such as deletions, induced in the cloned DNA might be transferred back to the influenza virus. Thus, it may be possible to develop stable mutants for experimental study and for use in immunoprophylaxis.

A procedure was devised for producing double-stranded DNA sequences corresponding to each of the influenza virus RNA segments. Negative and positive strands of influenza RNA segments were copied separately into DNA using the reverse transcriptase of avian myeloblastosis virus. Influenza virion RNA segments, which are negative strands, were reverse-transcribed into their complementary DNA copies in the presence of a specific priming DNA oligomer. The primer was complementary to the conserved 3'-end sequence of the virion RNA segments and the DNA products of the reverse transcriptase enzyme appeared to represent full-length genomic copies. Similarly, poly A-containing cytoplasmic RNA's (positive strands) isolated from the virus-infected cells were transcribed into DNA sequences in a reaction mixture in which oligo-(dT) primer was added. The single-stranded DNA molecules from both preparations were annealed to generate double-stranded DNA segments which were subsequently isolated for cloning in plasmid PBR322 of E. coli K-12.

The in vitro synthesized double stranded influenza DNA segments were then inserted into plasmid PBR322 at the specific Pst I site using the dG-dC tailing technique. The hybrid DNA molecules were used to transform recipient E. coli and transformants containing influenza gene sequences were identified by hybridization. In this manner, we have so far obtained putative full-length influenza virus DNA segments corresponding to genes coding for non-structural protein (gene VIII), matrix protein (gene VII), neuraminidase (gene VI), and hemagglutinin (gene IV). Cloning of other genes from wild type influenza A/Udorn/72 (H3N2) virus is now underway. Cloned DNA will be analyzed by restriction enzyme cleavage and nucleotide sequencing. By comparing such information for corresponding genes derived from influenza viruses belonging to different subtypes and different strains within a subtype it should be possible to define the genetic basis for variability of the hemagglutinin and neuraminidase antigens as well as some of the internal proteins (Lai, Markoff).

Respiratory Syncytial Virus

RS virus continues to be the major etiologic agent of bronchiolitis and pneumonia of early life, and the need for effective immunoprophylaxis was again emphasized last year by the occurrence of unusually large outbreaks of serious RS virus lower respiratory tract disease throughout the world.

Studies in Inbred Mice - Five animal models of RS virus infection have been studied by us, including the chimpanzee, cebus monkey, owl monkey, ferret and cotton rat. None of these species, however, is inbred, thus precluding genetic manipulation and certain types of immunologic study. Furthermore, few, if any specific immunologic reagents are available to allow study of certain aspects of RS virus pathogenesis and virus-host immunologic interaction.

In an effort to develop a model for experimental RS virus infection in an inbred species, we examined twenty strains of mice. Intranasal inoculation of RS virus in infant mice produced infection in each strain examined. However, there was wide variation among the inbred strains in the amount of virus recovered from the nose and lungs. The most resistant strain, CBA/CaHN, yielded only one-hundredth the quantity of virus recovered from the nose and lungs of the most permissive strain, DBA/2N. Ordering of geometric mean nasal and pulmonary titers from the twenty strains demonstrated a pattern of gradual, incremental increase from relatively resistant to relatively permissive strains. If level of viral replication were controlled by a single gene with few alleles, one would not expect to observe such a shallow linear array of titers. This suggests that response to RS virus infection is determined by a combination of genes, or perhaps a single gene with multiple alleles. Since there was no overlap between the virus titers observed for the strains that exhibited the lowest and highest levels of viral growth it should be possible to analyze the genetic control of viral replication using the appropriate crossbreeding techniques. In addition to the opportunity to study genetic control of viral replication, the availability of an inbred animal model for RS virus infection offers possibilities for studies which were not feasible previously. For instance, in vivo experiments using adoptive transfer of immunologic components, including immune cells, can now be performed. Another advantage of the mouse model, is the availability of a large number of specific immunological reagents that will permit us to examine individual portions of the immune system for their role in recovery from infection and resistance to infection and possible participation in immunopathology (Prince, Suffin).

Further in vivo Evaluation of ts-2 - Two seronegative chimpanzees were inoculated with a safety-tested ts-2 vaccine suspension prepared by Flow Laboratories. This vaccine is currently being evaluated in children. One animal was successfully infected in two attempts but did not develop any signs of illness, despite shedding a moderate amount of virus ($10^{4.2}$ pfu/ml of swab fluid) from the upper respiratory tract. The unavailability of further seronegative animals precluded additional study (Prince, Suffin).

In vivo Evaluation of ts-1 NG-1 and ts-1 NG-16 Mutants - The first ts mutant of RS virus to be tested in man, ts-1, appeared promising when evaluated in adult volunteers. However, tests in seronegative infants showed the virus to possess a low level of residual virulence, and to exhibit some genetic instability. In an attempt to further attenuate ts-1, nitrosoguanidine (NG) was used to remutagenize the ts-1 mutant. Two mutants, ts-1 NG-1 and ts-1 NG-16, were recovered from the progeny of NG treated ts-1 and were shown to exhibit greater temperature-sensitivity and genetic stability than ts-1. These two mutants were evaluated in the owl monkey because it is the only readily available experimental animal which develops clinically evident disease when infected with wild type RS virus. Neither ts-1 NG-1 nor ts-1 NG-16 differed significantly from wild type virus in either duration of infection or peak virus titer. However, the time of onset of virus shedding and the time of peak titer of both mutants were significantly delayed compared to wild type virus suggesting that both

mutants were, nonetheless, functionally defective. Finally, both mutants were significantly attenuated compared to wild type virus. These observations, in conjunction with previous studies showing ts-1 NG-1 and ts-1 NG-16 to be more defective and more stable genetically than the parental ts-1 mutant, suggest that they are potential candidates for use in a live vaccine. The fact that the two mutants did not differ significantly from each other in any of the observed parameters suggests that both should be subjected to additional in vivo testing in primates and, ultimately, man (Prince, Suffin).

Duration of Immunity Following Intranasal Infection of Cotton Rats - Man does not develop lasting immunity to RS virus infection. During the past 18 months the transient nature of RS virus immunity was investigated in cotton rats. Immunity to nasal infection was temporary; animals were susceptible to nasal reinfection beginning 8 months after primary infection. The level of virus replication in the nose increased with time after initial infection; at 18 months post-infection animals were completely permissive. In contrast, none of the animals examined throughout the course of the 18 month experiment were susceptible to reinfection of the lungs. If a similar situation obtains in man vaccination may offer greater promise of protection than had previously been considered possible (Prince, Suffin).

Intramuscular Immunization with Live RS Virus - Recently, Buynak reported that parenteral administration of wild-type RS virus, grown in human diploid cells, induced the development of serum neutralizing antibody in young children without causing any objective signs or symptoms of disease. These exciting findings clearly required amplification and extension, because evidence that virus replicated following intramuscular (IM) inoculation was not provided in the original report, nor was the possible immunosuppressive effect of maternally derived passive immunity evaluated. Following IM inoculation of $10^{2.2}$ to 10^{4} PFU live virus was not recovered from the local site and was never detected in the nose or lungs although extensive attempts were made over a prolonged period. Furthermore, attempts to detect viral antigens at the site of IM inoculation were unsuccessful. However, inactivation of infectivity of three strains of virus by the minimal UV dose markedly reduced or completely ablated their antigencitity and protective efficacy. Although this observation does not constitute unequivocal evidence for the occurrence of viral replication after IM inoculation, it suggests that limited replication, perhaps restricted to an abortive cycle, was responsible for stimulation of immunity by the small quantities of virus employed.

The possibility that passive immunity might interfere with the effectiveness of parenteral immunization with live RS virus was examined because immunosuppression could pose a serious obstacle to this approach. Thus, the greatest need for an RS virus vaccine is in the first few months of life, a time when infants possess a moderately high level of maternally derived RS virus serum antibody. We attempted to simulate these conditions by administering live RS virus IM to weanling rats possessing passive serum antibody derived from their immune mother. In this situation an immunosuppressive effect of

passive immunity was observed. Only 50% of inoculated weanling rats were rendered resistant to subsequent IN challenge with RS virus. This suggests that parenteral immunization with live virus may not be effective in protecting human infants against RS virus during their period of greatest vulnerability to serious RS virus disease, i.e., the first 3 months of life. If this be the case, the usefulness of live IM virus vaccine would be limited to individuals over 6 months of age who had escaped natural infection and who had lost most or all of their passive maternally derived serum antibody. The outlook for IM vaccination of individuals who had been infected previously is also not encouraging since Buynak's study indicated that seropositive children respond poorly to vaccine (Prince, Suffin, Chanock).

Immunopathoarcheology - We have been engaged in the development of a new method of enzyme-linked immunohistologic diagnostic technology. Because of the widely recognized difficulties in the evaluation of peroxidase staining due to endogenous peroxidase activity in tissues and inducibility of endogeneous peroxidase-like activity in inflammatory and neoplastic states a method of enzyme-immunohistochemistry was developed which would be unaffected by inflammatory and neoplastic processes. Our search for an enzymatic system not present in mammalian tissues led us to the consideration of glucose oxidase, an enzyme derived from non-mammalian sources, as a possible candidate for this purpose. Earlier work had shown that by modification of the reaction product a stable preparation could be obtaind suitable for immunohistochemistry. Antisera were prepared against this enzyme in rabbits, guinea pigs and goats. These antisera were converted into a soluble enzyme antibody complex and used in a manner similar to that described by Sternberger. Currently we are determining the critical variables for respiratory syncytial virus antigen preservation during the fixation and embedment processes. Subsequently an immunopathoarcheologic survey for RS virus and other viral pathogens will be performed using autopsy material collected over the past 20-30 years (Suffin).

Mycoplasmas

Efforts to assess the significance of pathogenic spiroplasmas are centered around the biological and serological characterization of organisms within this group, on development of appropriate models to explore pathogenicity and host response, and on evaluation of various techniques to determine the possible role of these agents in human disease. Experimental pathogenicity studies on chick embryos and one-day-old suckling rats with a number of spiroplasmas from diverse plant and insect origin have suggested a number of unique virulence markers in these organisms. Several tick-derived spiroplasmas (the SMCA group) are highly pathogenic for the chick embryo when as few as 1-10 spiroplasmas are administered, and most strains retain this virulence when passaged repeatedly on artificial medium. Spiroplasmas in this group demonstrated an inverse relationship between the mortality rate (LD_{50}) and the occurrence of cataracts in suckling rats. Strains which are highly virulent by intracerebral challenge to rats (when given 10^4 organisms or less) do not induce cataracts in survivors. At least one

strain (TP-2) in this group, exhibited a reversal in virulence pattern after repeated passage on artificial medium. While this organism showed a decline in pathogenicity for the chick embryo, there was enhanced virulence for the suckling rat. Additional studies of other spiroplasmas from insects and from free-living origins showed these organisms to have increasing pathogenicity for the chick embryo following repeated passage in the laboratory.

The new serological procedures developed in the Section for assessing the interrelationships of cultivated spiroplasmas has been applied with notable success. These techniques possess the necessary specificity and sensitivity to provide, for the first time, some understanding of those spiroplasmas possessing distinct antigenic determinants. A combined deformation/metabolism-inhibition test system, utilizing the activity of specific antiserum to deform helical organisms or inhibit metabolism of the organism, has been adapted to the microtiter system. The analysis of spiroplasmas has shown that the tick-derived SMCA group is serologically distinct from all other known spiroplasmas. In addition, two groups of spiroplasmas recovered from flowers, and the sex-ratio spiroplasmas from Drosophila species appear to be serologically distinct. The fifth serological group consists of a large number of plant and insect strains identical to, or partially related to, Spiroplasma citri. The results of this analysis, and the test itself, should provide a basis for continued sero-epidemiological study of spiroplasma antibody in man and other vertebrates. (Tully)

Honors and Awards

Robert M. Chanock

Director, International Reference Laboratory for Respiratory Viruses other than Influenza, World Health Organization, 1973 - present

Director, International Reference Laboratory for Mycoplasmas, World Health Organization, 1973 - present.

Editorial Board, Journal of Infectious Diseases

Associate Editor, American Journal of Epidemiology

Member, Advisory Board Archives of Virology

Invited lecturer, Johns Hopkins University, "Epidemiology and Prevention of Influenza", November 1, 1978

Invited lecturer, "Molecular Genetics of Viruses" course, FAES Graduate School, NIH, November 20, 1978

Invited participant, Royal Society Symposium on Influenza Virus Genetic, London, England, February 21 & 22, 1979

Invited speaker, Advances in Clinical Medicine, 1979, sponsored by the American Physicians Fellowship and the Foundation for Advanced Education in the Sciences, Bethesda, Maryland, March 29-30, 1979

Invited consultant, MRC Committee on Viral Vaccines, London, England. Invited to discuss future prospects for immunization against RSV disease, February, 1979

Chairman, Section on Medical Microbiology and Immunology, National Academy of Sciences

Appointed member Nominating Committee, National Academy of Sciences, 1979

Robert H. Purcell

Invited Participant and Co-chairman, US-Japan, Program Symposium on Viral Hepatitis, Tokyo, Japan, July 17-19, 1978.

Invited Participant, Emposium on Viral Hepatitis, Taipai, Taiwan, July 24-26, 1978.

Invited Speaker, Annual Meeting of The Infectious Diseases Society of America, Atlanta, Georgia, October 5-6, 1978.

Invited Speaker, 16th Annual Briefing on "New Horizons of Science", Council for the Advancement of Science Writing, Gatlinburg, Tenn., November 13-17, 1978.

Visiting professor, Michigan State University School of Medicine, East Lansing, Michigan, January 30, 1979.

Invited Speaker, "Update-Hepatitis", Sponsored by The New York Academy of Gastroenterology, New York City, February 26, 1979.

Invited Consultant, World Health Organization Consultation on Hepatitis B Vaccines, Geneva, March 12-14, 1979.

Invited Participant, International Symposium on Viral Hepatitis, Munich, April 5-6, 1979.

Invited Speaker, Symposium on Frontiers of Science and the Liver, Mt. Sinai Medical Center, New York City, June 6, 1979.

Albert Z. Kapikian

Invited member, International Coronavirus Study Group, 1972--
Invited member, Study Group on Reoviridae of International Committee for the Toxonomy of Viruses, 1976--
Invited member, Rotavirus subgroup of the WHO/FAO Comparative Virology Reoviridae Group, 1977--
Invited to serve as World Health Organization Temporary Advisor for Consultations on Rapid Laboratory Viral Diagnosis at Institute of Medical Microbiology, University of Gothenberg, Gothenberg, Sweden, Aug. 17, 18, 1978.
Invited to serve as one of the two vice-chairmen of Workshop "Aspects of Gastroenteritis Viruses" at the Fourth International Congress for Virology, The Hague, The Netherlands. Aug. 30 - Sept. 6, 1978. Workshop on Aug. 30, 1978.
Invited speaker at U.S. and Japanese Panels on Virus Diseases of the U.S.-Japan Cooperative Medical Science Program. Meeting at Osaka, Japan, Oct. 3-5, 1978.
Invited to become panel member of the United States Panel on Viral Diseases of U.S.-Japan Cooperative Medical Science Program, 1978-
Invited speaker at Children's Hospital, Washington, D.C., Infectious Diseases Conference, Nov. 3, 1978.
Invited to participate in a Workshop on Infectious Agents in Inflammatory Bowel Diseases at Tarrytown Conference Center, Tarrytown, N.Y., Nov. 17-19, 1978. sponsored by National Foundation for Ileitis and Colitis, Inc. and the American Gastroenterological Association. Selected as one of the members of the panel on cultivation of infectious agents for session III on Nov. 18, 1978.
Invited speaker at Preventive Medicine Symposium on Recent Developments in Infectious Diarrheas at 33rd Annual Meeting of The Society of Medical Consultants to the Armed Forces, Washington, D.C.
Invited to present a lecture of Virology '79 postgraduate course presented by Institute for Medical Research, Copewood, New Jersey. Presented lecture on Rotaviruses and the Norwalk Group of Agents on Jan. 11, 1979.
Invited to make brief presentation on viral diarrhea to Mr. Joseph Califano, Jr. Secretary of Health, Education and Welfare on March 8, 1979 in Dr. Donald Frederickson's office.
Invited to attend and to prepare a working paper on "Epidemiology of diarrheas caused by parvovirus-like agents" for the World Health Organization Scientific working Sub-Group Meeting on Rotavirus and Other Viral Diarrheas at the WHO Regional Office for the Americas, Washington, D.C., March 27-28, 19/9.
Elected chairman of WHO Scientific working Sub-Group Meeting on Rotavirus and Other Viral Diseases at WHO Regional Office for the Americas, Washington, D.C., March 27-28, 1979.
Invited co-convener of symposium Epidemiology of Enteric Viruses on May 9, 1979 at American Society for Microbiology Annual Meeting - U.S.-Japan Inter-society Microbiology Congress, Honolulu, Hawaii.
Invited to present lecture on Viral Diarrhea of Tropical Medicine Course on July 20, 1979 at Walter Reed Army Institute of Research, Washington, D.C.

Joseph G. Tully

Past-Chairman and Member, Board of Director, International Organization for Mycoplasmology (IOM)
Past-Chairman and Member of Board, IOM International Research Program on Comparative Mycoplasmology (formerly WHO/FAO Board)
Chairman, Subcommittee on Taxonomy of Mycoplasmas, American Society for Microbiology
Course Director, International Organization for Mycoplasmology Course on Mycoplasma Techniques, Bordeaux, France, September 3-21, 1979
Co-Director, Workshop on Detection of Mycoplasmas, Cell Science Center, Lake Placid, N.Y., November 16-20, 1978 and August 20-23, 1979
Member, International Subcommittee on the Taxonomy of Mollicutes
Invited speaker, Perspectives in Biology and Science, Iowa State University, Ames, Iowa, May 2, 1979
Invited speaker, Department of Microbiology, University of Bordeaux Medical School, Bordeaux, France September 14, 1978
Invited speaker, Bergey's Manual Roundtable, American Society for Microbiology, Los Angeles, Ca., May 5, 1979
Invited speaker, National Animal Disease Laboratory, Ames, Iowa, May 3, 1979
Invited speaker, Symposium on Mycoplasma Infection of Cell Cultures, Tissue Culture Association, Seattle, Washington, June 12, 1979
Invited speaker, Conference on Lethal Yellowing Disease of Palms, Fort Lauderdale, Florida, August 13, 1979.
Invited speaker, Conference on Current Status of the Agent of Contagious Caprine Pleuropneumonia, USDA, Hyattsville, Md. July 12, 1979

Brian R. Murphy

Invited speaker, Royal Society Symposium on Influenza Virus Genetics, London, England, February 21 & 22, 1979

Stephen M. Feinstone

Invited Participant, American Society for Microbiology Symposium on Epidemiology of Enteric Viral Infections. May 1979.
Invited Participant, Society for Epidemiologic Research Symposium on Viral Hepatitis, June 1979.
Invited Participant in a Symposium of the American Association for Tissue Banks, May 1979.
Invited Contributor to the Proceedings of a Symposium on Frontiers of Science and the Liver dedicated to Prof. Hans Popper

Harry B. Greenberg

Elected member, Infectious Diseases Society of America, 1979
Invited participant, American Society for Microbiology meeting, Los Angeles, Ca. and Honolulu, Hawaii, May, 1979

Richard G. Wyatt

Invited presentation on Viral Diarrhea to pediatric staff, housestaff, and medical students at Walter Reed Army Medical Center, Washington. D.C., February 12, 1979

Invited presentation on Viral Diarrhea to students participating in the Minority Biomedical Support Program, NIH, February 28, 1979

Consultant to Navajo Community College, Tsaile, Navajo Nation (Arizona) on Minority Biomedical Support Project: "Gastroenteritis in Newborn Lambs and Young Children on the Navajo Reservation", June 19-22, 1979

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00021-10 LID

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

THE ETIOLOGY OF ACUTE INFECTIOUS NONBACTERIAL GASTROENTERITIS

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Albert Z. Kapikian	LID,NIAID	Head, Epidemiology Section
	Dr. Harry B. Greenberg	LID NIAID	Medical Officer
	Dr. Anthony R. Kalica	LID,NIAID	Research Microbiologist
	Dr. Richard G. Wyatt	LID,NIAID	Medical Officer
	Dr. Robert H. Yolken	LID,NIAID	Research Associate
	Dr. Shigeo Matsuno	LID,NIAID	Guest Worker
Other:	Dr. Robert M. Chanock	LID,NIAID	Chief
	Mr. W. Lee Cline	LID,NIAID	Bio. Lab. Tech. (Micro)

COOPERATING UNITS (if any)

see next page

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

National Institute of Allergy and Infectious Diseases, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
155/12	55/12	100/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Objectives: (1) To search for viruses which play an etiological role in the syndrome of acute infectious non-bacterial gastroenteritis of infants, children, and adults; (2) to cultivate (in vitro) the viral agents of acute infectious nonbacterial gastroenteritis; (3) to study the biophysical, immunological and epidemiological characteristics of such agents; (4) to reproduce the syndrome experimentally for the purpose of a) studying the pathophysiological and immunological responses of the host, and b) assaying the infectivity of viruses such as human rotaviruses and the Norwalk and Norwalk-like agents; (5) to develop effective immunoprophylaxis for the viruses of acute, infectious, nonbacterial gastroenteritis; (6) to develop efficient and sensitive assays for a) detection of the viral and bacterial agents associated with gastroenteritis and b) antibodies to these pathogens.

PHS-6040
(Rev. 10-76)

Cooperating Units

Children's Hospital National Medical Center, Washington, D.C.; Johns Hopkins University, Baltimore, Md., Johns Hopkins University Center for Medical Research and Training, Dacca, Bangladesh, Division of Geographic Medicine of the Johns Hopkins University School of Medicine and the Baltimore City Hospitals, Baltimore Md.; Center for Disease Control, Atlanta, Ga.; Meloy Laboratories, Rockville, Md.; Plum Island Animal Disease Center, USDA, New York; New York Blood Center; University of Minnesota School of Medicine; University of Massachusetts; University of Virginia, Charlottesville; Bureau of Biologics, FDA; NINCDS; Veterinary Resources Branch, DRS; Cholera Research Laboratory, Dacca, Bangladesh; Royal Children's Hospital, Melbourne,Australia; Fairfield Hospital, Melbourne, Australia; INISA San Jose Costa Rica; INCAP, Guatemala; Tohoku University, Sendai, Japan; Department of Veterinary Science, Ohio Agricultural Research and Development Center, Wooster, Ohio; LBV, NIAID; LSD, NIAID; Duke University, North Carolina, University of New Mexico VA Hospital; Center for Vaccine Development, University of Maryland, Baltimore, Md.; UCLA; University of Rochester; University of Vermont; Walter Reed Army Institute of Research; California State Health Department, Berkeley, California; Children's Hospital, Philadelphia, Pa.; ICRR Sydney, Australia; University of Melbourne, Melbourne, Australia; Regional Virus Laboratory Ruchill Hospital, Glasgow, Scotland; WHO Collaborative Gastroenteritis Study involving numerous laboratories; Agricultural Research Council Institute for Research in Animal Diseases, Compton Newbury, Berkshire, England; Gorgas Memorial Laboratory, Panama; Medical Research Center, Nairobi; Department of the Royal Tropical Institute, Amsterdam, Holland.

Major Findings

Background - Important advances have been made in elucidating the etiological agents of acute infectious nonbacterial gastroenteritis, a syndrome that affects a broad segment of the population. It appears that viral gastroenteritis consists of at least two entities with distinct epidemiological characteristics. One, designated epidemic viral gastroenteritis, tends to occur in family or community-wide outbreaks affecting adults, school-aged children, family contacts and probably young children as well. The illness is usually self-limited and characteristically lasts 24-48 hours. In 1972, we demonstrated by immune electron microscopy (IEM) that a 27 nm particle Norwalk agent -- was associated with an outbreak of this form of gastroenteritis that occurred in Norwalk, Ohio. In addition, in further studies by our laboratory, particles that resemble the Norwalk agent morphologically have also been associated by IEM with 2 family outbreaks of gastroenteritis, one in Hawaii and the other in Montgomery County (MC), Md. The Norwalk and Hawaii agents appear to be distinct, whereas the Norwalk and MC agents appear to be related. An agent designated Ditchling or "W" has been associated with this form of gastroenteritis in studies in England.

The other form of gastroenteritis designated sporadic infantile gastroenteritis has been associated predominantly with a severe form of diarrhea affecting infants and young children, that often necessitates hospitalization and parenteral fluid therapy. It is evident now from studies in many parts of the world that the 70 nm human rotavirus is the major known etiological agent of sporadic infantile gastroenteritis. Before the discovery of this agent by Australian investigators in 1973, the etiology of the majority of cases of infantile gastroenteritis was unknown.

Our gastroenteritis studies are focused on the rotaviruses as well as on the Norwalk group of viruses. The Norwalk group of agents have not been cultivated conclusively in any in vitro system whereas the human rotavirus had been grown only in a limited fashion. However, in spite of the failure to grow the Norwalk agent, our studies have elucidated the importance of the Norwalk virus as a cause of epidemic viral gastroenteritis in various epidemiologic settings. In addition, a major breakthrough has been achieved recently with the successful efficient in vitro propagation of the human rotavirus. Highlights of research activities carried out with LID staff alone or in collaboration with others are outlined below.

In Vitro Cultivation of Human Rotavirus - A major effort of this laboratory has involved attempts to propagate efficiently human rotavirus. The efficient propagation of rotavirus type 2 appears now to have been achieved. New approaches were used in attempts to cultivate this agent. Initially, 42 fecal specimens known to be positive for rotavirus by EM were tested for their ability to infect primary African green monkey kidney (AGMK) cell cultures using low-speed centrifugation to enhance infectivity. Rotaviral antigens were detected by immunofluorescence (FA). Thirty-one of the 42 specimens were positive by this method but only 5

yielded as many as 10% FA positive cells. One strain ("Wa") detected in this manner was passaged subsequently 11 times in gnotobiotic piglets in collaboration with Dr. E.H. Bohl, Ohio Agricultural Research and Development Center, Wooster, OH. This approach was prompted by the findings of Provost and Hilleman who described the adaptation of hepatitis A virus to cell culture after 31 passages in marmosets. The occurrence of diarrhea was variable but piglets at each passage were infected based on finding viral antigens in the gut contents. After 11 passages in piglets, the "Wa" strain was passaged into primary AGMK cells by pretreating virus inoculum with trypsin (10 ug/ml) and centrifuging the inoculum at low-speed onto the cells. At that point the virus appeared to be capable of in vitro growth. Viral titers ranged from 1.1×10^3 fluorescent foci (FF)/ml to 3.9×10^6 FF/ml during the subsequent 14 passages. Contamination with SV5 was discovered at the eleventh passage (and retrospectively on earlier passages) but was removed by ether treatment. Beginning with "Wa" strain after six passages in AGMK, the virus has been plaqued and has undergone two plaque purifications.

Two approaches were used to establish the identity of this isolate as a human rotavirus. Polyacrylamide gel electrophoresis of viral RNA was performed; the RNA pattern of the isolate is identical to a previously recognized human rotavirus RNA pattern. It differs however, from six animal rotaviruses which grow readily in cell cultures, with which it was compared. Further, serological testing using plaque reduction and fluorescent focus neutralization was performed. The human "Wa" isolate appears to be serologically distinct from the bovine, porcine, and simian rotaviruses; it demonstrates a one-way antigenic relationship with the simian rotavirus SA-11--a finding consistent with a previous observation made by IEM by Schoub, et al.

Studies on the Etiology of Non-Bacterial Gastroenteritis Among Infants and Young Children Admitted to Children's Hospital, Washington, D.C. - These studies are carried out collaborative with Children's Hospital National Medical Center. Currently the stool or rectal swab specimens are examined by EM and ELISA at Children's Hospital for gastroenteric agents (Dr. Brandt) whereas the routine rotavirus serologic studies (CF) are carried out at the LID (Mr. Cline). The clinical data and specimens are furnished by Dr. Kim and her staff at Children's Hospital. In addition, the serotyping of rotaviruses has been performed at LID. The scope of the study was outlined previously and essentially involved study of pediatric patients admitted to Children's Hospital with a diarrheal illness. Limited outpatient studies were also performed.

In data analyzed by Dr. Brandt for the period Jan. 16, 1974 through June, 1978 the importance of rotavirus as the major etiologic agents of diarrheal illness was again highlighted. In last year's annual report we presented data from Jan. 1974 through May, 1978. The description in this annual report represents final analysis of data for the Jan. 1974 through June 1978 period. From January 16, 1974 through June 1978, specimens from 604 inpatients with gastroenteritis were tested by EM and ELISA for rotavirus and 232 (38.4%) were found to be positive. In contrast only 9 (1.7%) of 522 control inpatients studied March, 1974 through June 1978 were rotavirus positive by

identical techniques, thus demonstrating the statistically significant association of rotavirus infection with diarrheal disease ($p < .0001$). The pronounced predilection of rotavirus infection for the cooler months of the year was a consistent finding during this period. For example, the frequency of rotavirus infections in patients admitted to the hospital in January was striking as 87(71%) of 123 gastroenteritis patients shed this agent. Adenoviruses (as detected by EM and/or tissue culture) were associated with 5.1% of the diarrheal episodes, whereas 1.9% of the controls was adenovirus positive ($p < .05$) suggesting that these viruses play a role, albeit small, in the etiology of acute enteric disease. It was noteworthy that 26 of the 31 adenoviruses from inpatients did not grow when tested by routine tissue culture methods. Only 13 (2.2%) gastroenteritis inpatients were found to shed 27nm like virus particles (six also excreted human rotavirus), whereas 5(1.0%) of the controls shed this type of virus ($p > .05$) thus, indicating again that this group of agents is not an important etiologic agent of serious gastroenteritis in infants and young children requiring hospitalization.

From November, 1975 - June, 1978, rotaviruses were detected in 42 (21%) of 200 outpatients with diarrheal disease and in 8 (2.8%) of 290 outpatient controls. Thus, it appeared that rotaviruses caused not only severe diarrheal illnesses requiring hospitalization but also milder gastroenteritic illnesses which could be treated on an outpatient basis. Adenoviruses were found in 2.5% of outpatients with gastroenteritis and in 0.3% of outpatient controls ($p < .05$) again supporting the contention that adenoviruses may play a role in gastrointestinal disease. 27 nm particles were found in 1 outpatient with gastroenteritis and in 2 controls.

Approximately three fourths of the rotaviruses detected were found to be type 2 which was prevalent during 5 successive epidemic years from Jan. 1974 through June 1975. Type 1 was detected in the last 4 successive epidemic years and comprised 46% of the human rotavirus infections detected among gastroenteritis inpatients during the year 1977-78. Numerically, human rotavirus infection was detected most often in those gastroenteritis inpatients who were 10 through 12 months of age. The group of gastroenteritis inpatients with the highest percentage of human rotavirus infection was 13 through 15 months of age. The excess of type 2 human rotavirus infection in relation to type 1 infection was especially large in those aged 7 through 24 months. Lower socioeconomic status or greater crowding appeared to be associated with the occurrence of rotavirus infection earlier in life and earlier in the epidemic year.

More up to date data (up to April, 1979) on the role of the above agents (minus new serotyping data which is not available) analyzed by Dr. Brandt are consistent with the previous years' experiences.

Incidence of Pediatric Rotavirus Gastroenteritis Resulting in Admission to Hospital in Washington D.C. Area -In studies carried out by Dr. Rodriguez at the Children's Hospital National Medical Center, Washington, D.C., the incidence of rotavirus gastroenteritis requiring admission to this hospital

in a defined population 15 yrs. of age and under was determined. This was a major achievement as the importance of rotavirus as a cause of pediatric gastroenteritis has been ascertained primarily in studies of infants and children hospitalized with this disease. However, in such studies, incidence rates of hospitalization caused by rotavirus infection could not be derived since it was not possible to define precisely the population from which the hospitalized patients came. Thus, the incidence of rotavirus gastroenteritis requiring hospitalization was estimated for a defined population of approximately 105,000 individuals whose health care was provided by Group Health Association (GHA), a health maintenance organization in the Washington, D.C. area. About 28% were 15 yrs. of age or under. Almost all of the pediatric hospitalizations of this health maintenance organization occurred at the Children's Hospital. Visits to the GHA clinics for diarrhea were recorded but those illnesses were not studied for viral etiology. However, etiologic studies were performed for GHA infants and children who were admitted to Children's Hospital for acute gastroenteritis. Data on this population was studied for the period Jan., 1977 through March, 1979, an interval encompassing three periods of rotavirus prevalence. Approximately 29,000 individuals 15 yrs. of age and younger comprised the study group for each period. On the average, 1 in 272 (3.7/1000) infants under 1 yr. of age and 1 in 451 (2.2/1000) 13-24 months of age were hospitalized for rotavirus disease in each period of rotavirus prevalence. The incidence of rotavirus gastroenteritis requiring hospitalization precipitously after 2 years of age and such illness requiring hospitalization was not detected after 5 years of age. The role of other agents in acute gastroenteritis requiring hospitalization was minimal compared to that of rotavirus. It was striking that when hospital admission patterns for gastroenteritis of any etiology were examined by month, 76% (28/38) of such admissions in the study population occurred during the winter months of January, February and March. The data derived from studying those hospitalized from the GHA population suggest that at least 50% of admissions for dehydrating gastrointestinal illness was associated with rotavirus infection.

Study of Rotavirus Infection in Adult Volunteers - Data from the initial phase of this study, reported in a preliminary fashion last year, is now complete. A safety tested inoculum of rotavirus type 2 consisting of a dilution of a 2% stool filtrate prepared from a stool rich in rotavirus particles obtained from a 13 month old child hospitalized with gastroenteritis was administered orally to 18 volunteers once and to 2 of these volunteers a second time, about 19 months after initial challenge. These studies were carried out in 6 phases; the first at the Clinical Center, NIH and the remainder at the University of Maryland Center for Vaccine Development in Baltimore, Md. The first study began in November, 1974 and the sixth in June, 1978. In the initial study, 2 volunteers with high levels of serum rotavirus antibody were selected since the clinical response to rotavirus infection under experimental conditions was not known. Since illness did not occur, 2 additional volunteers with somewhat lower levels of rotavirus antibody were inoculated in a second study; neither developed illness. Efforts were then made to identify volunteers with little, if any, rotavirus serum antibody. It soon became apparent however, that almost all

adults possessed such antibody detectable by at least one of the assays employed. Thus, when feasible, individuals with the lowest serum antibody titers were selected for inoculation. Five of 18 volunteers shed rotavirus in their stools beginning on the second, third or fourth day after inoculation. Although only 5 shed rotavirus, 15 of the 18 developed a four-fold or greater increase in serum antibody to rotavirus as detected by at least one of the assayed employed. ELISA was the most efficient of the serologic assays. Four of the 5 volunteers who shed rotavirus developed a diarrheal illness which began 2-4 days after inoculation. The average duration of diarrhea was 2.5 days with a range of 1-4 days. Two of these 4 volunteers also vomited.

Although there was a tendency for detectable preinoculation serum antibody as measured by CF, IF and neutralization (of NCDV) tests to be associated with resistance to diarrheal illness following rotavirus inoculation, the trend was not statistically significant. Analysis of the relationship between serum antibody detectable by these methods and the occurrence of rotavirus shedding revealed a significant association only for detectable IF antibody although a similar trend was again observed for the other assays. Thus, by selecting individuals with the lowest levels of serum rotavirus antibody, an attack rate of rotaviral diarrhea disease of 38-60% was achieved. Similar analyses were made to evaluate the protective effect of pre-existing rotavirus type specific serum IgG antibody by ELISA: a significant association was not observed for the presence of either type 1 or 2 serum IgG antibody and failure to shed virus or develop diarrheal illness.

However, each of 5 volunteers without detectable rotavirus type 2 intestinal fluid IgA antibody shed virus indicating that the presence of local IgA rotavirus antibody correlated with resistance to virus shedding ($p < .05$). Moreover, 4 of 5 volunteers without detectable IgA rotavirus antibody in intestinal fluids, but none of 10 with such antibody, developed a diarrheal illness after inoculation ($p < .05$) suggesting that the presence of such antibody was associated with resistance to diarrheal illness. Rotavirus types 1 IgA intestinal fluid antibody did not correlate with resistance to virus shedding or illness. Thus, it appears that local intestinal IgA rotavirus antibody correlates well with resistance to rotavirus shedding and diarrhea under experimental conditions. In addition, 4-fold IgA antibody increases to type 2 rotavirus were observed in intestinal fluid from each of the 5 volunteers who shed rotavirus, and from an additional volunteer as well, following initial challenge.

Two volunteers who developed illness and laboratory evidence of rotavirus infection following initial administration of rotavirus type 2 were given the same inoculum 19 months later. Neither developed a diarrheal illness or vomiting after second inoculation although one developed mild clinical manifestations. Both individuals lacked detectable rotavirus IgA intestinal antibody prior to initial challenge but had such antibody prior to second challenge. One (who had the mild clinical manifestations) developed a 4-fold increase in intestinal IgA rotavirus antibody after the second inoculation as well.

Frequency of Detection of Rotavirus and Norwalk Agent in Stools of Infants and Children with Gastroenteritis in World Health Organization Collaborative Study - In a WHO collaborative study, specimens were obtained from pediatric patients with diarrheal illnesses from various parts of the world including: Dakar, Senegal; Entebbe, Uganda; Singapore; Tunis, Tunisia; Kalubowila, Sri Lanka; Seoul, Korea; Kuala Lumpur, Malaysia; Hong Kong; Cayenne, French Guiana; Bungui, Central African Republic; and Kinshasha, Zaire. Specimens were obtained Feb., 1976-July, 1978. Dates were not available for the last 2 locations. A total of 438 specimens were examined for rotavirus by ELISA and 118 (27%) were positive. Rotavirus was detected in each of the countries with a range of 7% to 71% being positive. In contrast, these stool specimens were also tested for Norwalk antigen by RIA and none was positive.

Antibodies Against Rotavirus in Sera from Children Living in the Machakos District of Kenya - In a collaborative study with Metselaar, Sack, and Muller the prevalence of rotaviral CF antibody in children under 5 years of age living in the Machakos District of Kenya was determined. Two different antigens, NCDV and "O" were used and the superiority of "O" antigen over NCDV was confirmed. The percentage of children with rotavirus antibody rose rapidly so that by 18-23 months of age 79% had such antibody indicating that the time of initial rotavirus infection in the Kenya population was similar to that observed in various other areas of the world.

Plaque Reduction Tests with Animal Rotaviruses - Plaque reduction tests to measured serum antibody against bovine rotavirus (UK strain) and simian rotavirus (SA11) were performed regularly to characterize immune response, identify specific hyperimmune sera and to identify rotavirus isolates such as those arising from genetic reassortment studies. While other rotavirus strains (porcine, bovine-NCDV, and "O" agent) have been plaqued, plaque reduction tests have not been performed.

Development of ts Mutants of Calf Rotavirus - As part of our effort to modify the virulence of rotavirus, 8 ts mutants of the UK strain of calf rotavirus were developed using 5 azacytidine or nitrosoguanidine as mutagen. The 8 mutants had a shutoff temperature for plaque formation of 38°C or 39°C and appeared to be relatively stable genetically. It is our intention to use these and subsequent mutants to probe the function of individual genes of the rotavirus genome. Ts mutants will also be used as donors of ts genes for transfer to the tissue culture-adapted human virus. Bovine genetic reassortment in an attempt to attenuate the human virus. Bovine rotavirus genes will probably prove to be host restricted in human cells, i.e., hr, and this may offer another mechanism by which genes transferred from the bovine virus could bring about attenuation of the human virus.

Animal Rotavirus as Potential Vaccine for Humans - The evaluation of the ability of bovine rotavirus to protect against subsequent challenge with human rotavirus was presented in a preliminary way in the FY 1978 annual report. In the past year this study has been finalized and published in Science. In the first phase of the study 2 calves were infected in utero with bovine rotavirus (Cody strain from Nebraska) and challenged with homologous virus

shortly after birth. Complete resistance to disease on this second challenge was observed, while two control animals which were not infected in vitro but challenged shortly after birth became ill. For the second phase, 5 animals were infected with bovine rotavirus in utero and challenged with the heterologous human rotavirus (type 2) one or two days after birth. All animals were resistant to disease in contrast to eight control animals which received human rotavirus one or two days after birth without having been exposed previously to bovine rotavirus; seven of the control animals became ill. These data suggest that the bovine virus is sufficiently related to the human type 2 virus to warrant further evaluation of the former as a live vaccine.

Further studies on the bovine rotavirus (UK strain) are under way to ascertain its suitability as a possible vaccine candidate for human use. This bovine rotavirus strain was chosen because it has been cultivated only in primary bovine kidney cell cultures and would likely meet the requirements for human use. It is serologically closely related to the bovine rotavirus (Cody) from Nebraska which was evaluated in the animal cross-protection studies, although it does not exhibit an identical RNA gel electrophoresis pattern. A large suspension (approximately 2 liters) of this plaque-purified bovine rotavirus underwent successful routine safety-testing. Additional safety testing is being carried out at Plum Island Animal Disease Center in collaboration with Dr. C.A. Mebus. This is required since this particular rotavirus strain originated in the UK from a domestic animal. No adventitious agents have been recognized in the virus suspension, and the disease it produces in calves is similar to that seen with the U.S. bovine rotavirus. However, the UK isolate does induce disease in gnotobiotic piglets (4 of 7) while the U.S. isolate does not. When testing is complete, USDA will be asked for permission to release this strain for distribution and further study.

Nonhuman Primate Animal Models for Gastroenteritis - A further attempt to induce illness with human rotavirus (type 2) in 2 young seronegative chimpanzees failed. Both animals were infected based on virus shedding in feces. The evailability of a satisfactory nonhuman primate model for rotavirus disease still remains a goal.

An attempt to infect 2 chimpanzees with the 27 nm Marin County agent failed. An IEM antibody response was not observed (see later).

Analysis of Rotaviral RNA by Gel Electrophoresis - Continued use was made of the technique of electrophoresis of rotaviral RNA for detection of differences and/or similarities among various animal and human strains. It was evident from previous analysis of 7 type 2 human rotaviruses that three different RNA patterns existed. Since only one ELISA serotype 1 strain was included in the original studies, a second serotype 1 strain (# 268 from Bangladesh) was compared with the original type 1 strain (DS-1 from Children's Hospital); 5 of the DS-1 segments had a different migration rate from the corresponding gene segments of strain 268. Thus, we detected at least two RNA patterns for ELISA serotype 1 human rotaviruses.

Several calf rotavirus strains from Canada (RS,C10,C27 and C486) were grown in cell culture and RNA was prepared for comparisons among the calf rotaviruses. Two of these (RS and C10) did not grow well enough to permit further study. Of the remainder, C27 appeared to be identical to NCDV by RNA analysis

and the second (C486) appeared to differ in migration of at least one segment (#5) from NCDV. This strain (C846) was contaminated by a second virus, since its RNA pattern contained a second less abundant #5 segment. Both Canadian calf viruses had an RNA pattern different from that of the UK calf rotavirus pattern.

Since there are two isolates of Nebraska calf diarrhea virus (NCDV), namely the Cody and Lincoln isolates, a comparison of their RNA patterns was made to see if they could be distinguished on this basis. Results from the comparison showed them to be indistinguishable.

RNA analysis was also used to characterize a putative human rotavirus that grew in cell culture (described earlier). Comparison of this isolate (Wa strain) with representatives of two of the previously described human rotavirus patterns ("D" strain, Group I and "L" strain, Group II) were carried out. The "Wa" RNA pattern was identical to that of "L" strain and differed from that of the "D" strain by the migration of segment #5. The Wa isolate was further shown to be different from the following animal rotavirus RNAs by difference in migration of 3 to 7 of the 11 segments: SA-11, "O" agent, OSU and EE porcine rotaviruses,NCDV and UK calf rotaviruses. Thus, the RNA analysis showed that Wa is most likely a human rotavirus.

In addition, the methodology for preparing individual rotavirus genes or segments was developed. 50 to 150 ug quantities of total RNA was prepared from cell culture grown animal rotavirus strains and examined by gel electrophoresis. After gel electrophoresis, separated segments are cut out of the gel. In this manner, 1 to 5 ug quantities of segments #1, 4, 5, 6, 10 and 11 have been readily prepared from SA-11, NCDV and O agent RNAs. Since they do not resolve well by electrophoresis, mixtures of RNA segments 2 and 3 as well as segments 7,8 and 9 have also been prepared from these three rotaviruses. The segments have been examined by electron microscopy and are intact, although some RNA breakage has occurred. This technology will be useful for translation experiments using a reticulocyte system, and S-35 methionine.

Rotavirus Hemagglutination (HA) and Hemagglutination-Inhibition (HI) Studies - Cell culture grown simian rotavirus (SA-11) and calf rotavirus (C486 from Canada) have been established as hemagglutinating rotavirus strains. Other cell culture adapted animal rotavirus isolates were tested for their ability to hemagglutinate. The virus preparations were genetron treated to remove possible inhibitors and concentrated 50-fold before being assayed in a phosphate buffered saline HA system. SA-11 was used as a positive control and gave optimal HA activity with Rhesus monkey red blood cells. NCDV and "O" agent were found to hemagglutinate Rhesus monkey and guinea pig cells, whereas the UK calf virus and OSU porcine strain were HA negative. Rhesus monkey erythrocytes were chosen for routine HA assays since they worked as well as human "O" cells and were available commercially.

Shinozaki and coworkers have published results which demonstrate the existence of a human rotaviral hemagglutinin and subsequently have indicated

that Tris buffered saline supplemented with Ca and Mg ions, and chicken erythrocytes provide optional hemagglutination activity. Other workers in Japan (Sato et al. and Inaba et al.) have used Tris-HCl buffered saline and veronal buffered saline with chicken cells to demonstrate HA activity for NCDV. The Tris buffered saline and veronal buffered saline were used with chicken cells and monkey cells in our laboratory to assay various preparations containing human and animal rotaviruses.

Fifty-fold concentrates of the cell culture adapted animal strains and 12 human rotavirus suspensions prepared by genetron treatment and 5-fold concentration of gnotobiotic calf feces collected during experimental infection were assayed for HA activity by the two alternative HA systems mentioned above. The human rotavirus preparations represented first through third passage of 3 different strains ("D," "C" and "DS-1") in calves. A human hemagglutinin could not be demonstrated by these methods and those animal strains which were negative by the routine method used in our laboratory were also negative in these systems. Compared to our standard method, the Tris buffered saline system gave lower titers and the veronal buffered saline system gave negative assays using the HA positive viruses, SA-11, NCDV and "O" agent.

It was shown by others (Cohen et al., Hruska et al. and Estes et al.) that EDTA removes the outer capsid of rotavirus. A preliminary experiment was carried out to determine the effect of EDTA on the HA of NCDV and SA-11. SA-11 and NCDV HA appeared to have different sensitivities to EDTA. EDTA at concentrations above 0.5 mM destroyed NCDV HA activity, whereas SA-11 HA was reduced only 2 to 4-fold at concentrations of 1 to 5 mM of EDTA.

The fifty-fold concentrates of SA-11, NCDV and "O" agent were used as antigens in HI tests with 23 human serum pairs from children ill with diarrhea (Children's Hospital). 9 of the 23 children had developed an 4-fold increase in rotavirus CF antibody and each of the 23 had demonstrated a significant rotavirus type specific antibody response by ELISA; 11 were type 1 and 12 were type 2. Six of the 23 demonstrated a significant HI antibody rise to SA-11 and none developed an antibody response to either NCDV or O agent. Four of the six with HI responses had developed antibody responses by ELISA to type 2, and two to type 1 rotavirus. When compared to CF, 2 of the HI rises occurred in patients who did not develop a significant CF antibody rise, but 2 who were negative by HI were positive by CF, and 2 were CF and HI positive.

Hyperimmune guinea pig sera to SA-11 and NCDV were prepared by using crude infected cell culture fluid mixed with Freund's imcomplete adjuvant as immunogen. These sera did not cross react with each other by HI and had a homologous titer of 1:512. Hyperimmune anti- O agent serum was made in this manner, but in one way tests with SA-11 and NCDV the "O" agent cross-reacted with the anti-NCDV serum but not with the anti-SA-11 serum.

Six guinea pig sera and one goat hyperimmune serum prepared against doubly purified (sucrose and CsCl gradients) human rotavirus failed to show HI activity against any of the three HA antigens. However, one other

goat (930) which was hyperimmunized with sucrose purified human rotavirus developed HI antibody to all three viruses; also human immune serum globulin (Armour Corp.) had HI activity against all three antigens at a titer of 1:32.

Studies of Rotavirus Polypeptides - African green monkey kidney cells were infected with an MOI of 10 of SA-11 virus and at intervals from 2 to 4 hours post infection were pulse labeled for 2 hours with S-35 methionine. Cytoplasmic extracts were prepared according to the methods employed by Ramig et al and electrophoresed on discontinuous tris-glycine gels. With this technique, seven viral polypeptides were detected and maximum polypeptide synthesis appeared from 12 to 18 hours post infection. A high background of labeled cellular components was a distinct problem and probably masked viral polypeptides which were present in small quantity. To overcome this, hyperimmune anti-SA-11 and anti-NCDV guinea pig antisera (as described in section on HA) were prepared and will prove useful for immunoprecipitation of these less abundant polypeptides.

Experiments were carried out to label the structural polypeptides of SA-11, UK calf, and NCDV with S-35 methionine produced during infection of African green monkey kidney or CV-1 cells. The virus was harvested and purified on shallow CsCl gradients. Density and HA activity were used to assay for complete virions and core particles for the purpose of determining the inner and outer capsid polypeptides for each of the three viruses. However, NCDV and UK complete virions appear to be sensitive to the combination of CsCl and centrifugation since no detectable complete virions were detected in CsCl gradients despite a relatively high HA titer of the NCDV starting material. SA-11 appeared to be more resistant to centrifugation in CsCl since HA positive labeled particles were detected at densities of 1.35 and 1.36 g/cc. Gel electrophoresis of these preparations was carried out on a discontinuous tris-glycine system for comparison with the continuous phosphate buffered system used in earlier studies. The results with S-35 labeled SA-11 in the continuous gel system closely resembled those achieved in our earlier work using a protein staining technique, i.e., 8 polypeptides were seen for the HA positive SA-11 fraction. The UK and NCDV fractions yielded only 5 polypeptides in the continuous gel system indicating that they were composed of labeled core particles. Gel electrophoresis of these samples in the discontinuous system gave fewer and less well resolved polypeptides for the SA-11 virions (i.e., 7 polypeptides) and about the same results for the core SA-11, NCDV and UK particles (i.e., 5 polypeptides).

Role of Norwalk Virus in Outbreaks of Non-Bacterial Gastroenteritis - The development of a radioimmunoassay (RIA) for detection of Norwalk antigen and a radioimmunoassay blocking (RIABL) test for detection of antibody to the Norwalk agent were described in the last two years' annual reports. The availability of the RIA and RIABL has permitted continued large scale epidemiologic studies of Norwalk infection.

The original study of epidemic gastroenteritis has now been considerably enlarged. 65 outbreaks have been studied serologically. and 20 (31%) appear to

be associated with the Norwalk virus as indicated by seroresponse of affected individuals. This information reaffirms our previous conclusion that the Norwalk virus is a major cause of epidemic gastroenteritis. The new Norwalk-associated outbreaks occurred in a wide variety of situations including grade school, summer camp, nursing home, and cruise ship. Interestingly, Norwalk virus was etiologically associated with a very large oyster borne outbreak of acute gastroenteritis in Australia by Australian investigators and confirmed in our laboratory. In two Norwalk-associated outbreaks originally studied by the CDC, water borne transmission appeared to be the mode of spread.

Antibody Prevalence to the Norwalk Virus - A large battery of age stratified sera from adults from various parts of the world was studied for Norwalk antibody. Prevalence rates were stable and similar in adults from the U.S., Switzerland, Belgium, Yugoslavia, Ecuador, Bangladesh, and Nepal (approximately 70%). On the other hand, children from the U.S. and Yugoslavia acquired antibody far more slowly than did children in Ecuador and Bangladesh. In Ecuador and Bangladesh an antibody prevalence of greater than 70% was found in children 0-5 years of age. This high childhood prevalence rate may indicate that Norwalk virus plays an important role in childhood gastroenteritis in underdeveloped countries. The low level of antibody prevalence in young children from the U.S. is in keeping with the failure to incriminate this agent as a major cause of infantile gastroenteritis in the U.S.

Norwalk Virus and Traveler's Diarrhea - In collaboration with R.B. Sack and others we have investigated the role of Norwalk virus in traveler's diarrhea. Serologic studies were performed on sera from Peace Corps volunteers in Kenya, Morocco, and Honduras. These volunteers were involved in doxycycline efficacy studies. There were no seroresponses detected in Peace Corps workers in Kenya. In Honduras and Morocco, a small percentage of ill individuals (8-9%) had evidence of Norwalk infection. (Three individuals in the Morocco study had serologic evidence of Norwalk infection but did not develop diarrheal illness; in addition, only one individual in the Morocco study had a significant increase in rotavirus antibody but did not develop diarrheal illness.) These findings imply that the Norwalk virus is one of the minor causes of tourista in American travelers. In an interesting study of tourista in Panamanians traveling to Mexico, Norwalk virus infection was also associated with traveler's diarrhea (15%). In this study, rotavirus, Norwalk agent and Campylobacter were more important than toxigenic E coli as possible causes of traveler's diarrhea. The study of Panamanian tourista was done by R. Ryder and others.

The Role of Norwalk Virus in Enteric Gastroenteritis - Prospective Family Studies - In collaborative studies with Dr. W. Rodriguez, Children's Hospital, Wash., D.C., Dr. Mark Gurwith, UCLA Medical School and Dr. R. Guerrant, Univ. of Virginia, we studied the incidence of Norwalk infection in families followed prospectively for diarrheal disease. Preliminary data from the Rodriguez and Guerrant study indicate that the Norwalk agent might be associated with between 6 and 30% of family episodes.

It is hoped that in the coming months further analysis of this serologic data will better define the role Norwalk virus plays as a cause of endemic

sporadic infectious diarrhea in both children and adults in the U.S. and Canada. Similar prospective studies of families in underdeveloped countries are needed.

Detection of Norwalk Virus in Vomitus - By RIA, we examined 5 vomitus specimens obtained from 5 volunteers experimentally infected with the Norwalk virus. The vomitus specimens were obtained during acute illness, 24-48 hours after inoculation. Four of the 5 specimens were positive for Norwalk antigen by RIA. Following 100-fold concentration of one of the specimens, the Norwalk particle was visualized by IEM.

Anti-viral Therapy for Norwalk Disease - In a collaborative study with Dr. R.G. Douglas in Rochester, we evaluated the therapeutic efficacy of Bismuth Subsalicylate (BSS -- Pepto-Bismol) for Norwalk gastroenteritis. BSS had previously been shown to be an effective therapy for diarrhea caused by toxigenic E coli. In a volunteer study involving 59 volunteers, BSS was shown to have a minor but significant effect on the course of Norwalk virus gastroenteritis reducing both the severity and duration of cramps but not affecting viral shedding.

Serologic Tests for Relatedness of the Norwalk Virus and Other Gastrointestinal Viruses - As part of continuing effort to find other viruses serologically related to the Norwalk agent, we tested antisera raised to two new candidate animal gastroenteritis agents that morphologically resemble Norwalk. The Newberry agent (a calf agent from Dr. J. Bridger, England) and several dog parvo- and calicivirus isolates (Dr. L. Binn, Walter Reed) were examined and found to be antigenically unrelated to Norwalk virus. In addition, antisera to an astrovirus (Dr. McSwiggan, England) and several established caliciviruses did not react with Norwalk virus.

Norwalk and Hawaii Volunteer Studies - 23 volunteers were administered the 8FIIA Norwalk inoculum. These studies were carried out basically for two reasons. Jejunal secretions were gathered so that the local immune response to the Norwalk virus could be studied. As expected, approximately half of the inoculated volunteers became ill. Using a sensitive RIABL,we could find no correlation between preexisting serum or intestinal fluid antibody titer and protection. We also found that a subset of individuals clinically resistant to Norwalk illness appeared to be absolutely refractory to infection, i.e., they have no detectable antibody prior to or after challenge with virus. These findings may indicate that a proportion of people are resistant to Norwalk virus on a genetic or physiologic rather than immunologic basis. A similar finding had been made by Parrino et al in Boston in a collaborative study and was described in a previous annual report.

Using stool specimens obtained from the volunteer studies, we attempted to purify whole virion and a soluble viral protein found in stool. Norwalk particles are present in small amount in stool so that progress has been slow. Using a combination of isopycnic and rate zonal ultracentrifugation, iodination and polyacidamide gel electrophoresis, we have preliminary evidence showing the Norwalk particle contains 3 proteins,

2 approximately 70K and 1 of 40-50K. These findings most closely resemble that described for parvoviral proteins. In addition, a soluble viral protein was found in stools from volunteers infected with Norwalk virus. Using gel filtration, ion exchange chromatography and affinity chromatography we have partially characterized this protein. It has a molecular weight of approximately 45K and antibody to it agglutinates whole virus.

We recently carried out one volunteer study with the Hawaii agent. 7 of 12 volunteers became ill. Jejunal secretions were gathered and will be analyzed in the future. Stool and vomitus specimens are currently being looked at by IEM in order to identify particle positive specimens. These will be used in the development of a Hawaii RIA.

Other Agents - Another 27 nm particle, the Marin Co. agent (kindly supplied by Dr. L. Oshiro) was studied. This agent was clearly associated with an outbreak of gastroenteritis in a nursing home in California. It is not serologically related to Hawaii or Norwalk agents. We have given a stool filtrate preparation of the agent to two chimpanzees. As noted before, neither became ill or seroconverted by IEM. We are currently safety testing this filtrate for future studies in volunteers.

Development of a Solid-Phase Microtiter Radioimmunoassay Blocking Test for Detection of Antibodies to Escherichia coli Heat-Labile Enterotoxin - A solid-phase microtiter radioimmunoassay for the detection of E coli heat-labile enterotoxin was described in a previous annual report. This assay was modified into a blocking test to detect antibodies to E coli heat-labile enterotoxin, using burro antiserum to cholera toxin and IgG fraction of this serum as cross reactive antibody to E coli heat-labile toxin.

A comparison of the efficiency of the RIA blocking and the adrenal cell neutralization tests for detecting serological responses in volunteers experimentally infected with toxigenic E coli revealed that both techniques were efficient at detecting rises in ill volunteers. (The volunteer studies had been carried out by Dr. M. Levine et al of the University of Maryland.) Nine of 10 volunteers who became ill developed an RIA blocking antibody response, and 7 had a serological response when tested by the adrenal cell method. Proportionately fewer non-ill volunteers responded when tested by either method. The adrenal cell assay detected one more seroresponse in the non-ill volunteers than did the RIA. RIA blocking antibody titers were between 5- and 10-fold higher than adrenal cell titers, and preexisting antibody was detected somewhat more commonly by the RIA blocking method.

We next compared the efficiency of the two techniques for detecting seroresponses using paired sera from adults with naturally occurring diarrhea in Kenya and Bangladesh. The results of the two tests were similar. Both methods detected significant rises in antibody in just under one-half of the patients shown to be shedding toxigenic E coli (5 of 12; the same 5 individuals developed a response in both assays). Of 14 patients with acute diarrhea who did not have detectable toxigenic E coli in their stools, 2

had a significant antitoxin rise by RIA blocking test and 1 of these also developed a rise by adrenal cell neutralization test. Again the actual antibody titer of these sera was considerably higher in the RIA blocking test than the adrenal cell neutralization assay; however, the relative correlation of the two tests was high. No change in titer was detected with the RIA blocking test when four paired sera from volunteers infected with Norwalk agent or four paired sera from children with naturally acquired human rotavirus infection were tested.

The age prevalence of serum anti-LT was investigated by screening random sera obtained at Children's Hospital, Washington, D.C. and Junior Village over the past 15 years. By 48 months of age, over 50% of the population studied had developed antibody to E coli LT. In addition, it was found that over 70% of a group of University of Maryland students aged 17 through 26 had detectable serum anti-LT.

Because the RIA blocking technique appeared to readily detect anti-LT in pediatric sera, we studied the acute and convalescent bloods obtained from a group of children with acute diarrhea seen at Children's Hospital, Washington, D.C. This group of patients was a subset of a previously studied population. They were patients in whom, despite extensive investigation, human rotavirus could not be implicated as a cause of their diarrhea. None of these 51 individuals showed evidence of a serological response to E coli LT.

Development of an ELISA for Detection and Identification of Coxsackie A Viruses - Many of the Coxsackie A viruses - a group consisting of 23 serotypes - have been shown to cause a wide variety of diseases. However, many strains grow poorly if at all in conventional cell cultures and require suckling mice for their propagation. The difficulties in using suckling mice has hampered the study of Coxsackie virus infections. We recently developed an ELISA for detecting and serotyping Coxsackie A viruses using Coxsackie virus type specific monkey sera as precoat (capture antibody), type specific mouse sera as second antibody (detector antibody) and goat anti-mouse globulin conjugated with alkaline-phosphatase as indicator antibody. ELISA was considerably more sensitive than CF for detecting Coxsackie virus. In addition, by ELISA we were able to correctly type strains of all 23 recognized serotypes of Coxsackie A virus. With 22 of the 23 viruses significant positive ELISA reactions were noted only with homotypic sera. Some cross reactivity was found with Coxsackie A-12 virus. Coxsackie A-12 antigen had small amounts of ELISA reactivity with Coxsackie A-5 and A-7 antisera. However, the Coxsackie A-12 virus cound be distinguished from the other two types because of the greater P/N value of the homologous reaction.

Significance to Biomedical Research and the Program of the Institute

Acute infectious nonbacterial gastroenteritis is a common infectious disease which affects a broad segment of the population and was the second most common disease observed in a 10 year family study in the United States. In addition, diarrheal diseases are a leading cause of mortality as well as morbidity in

developing countries. Ultimate goals of prevension and therapy have been furthered in our current studies by (1) the detection and preliminary characterization of etiologic agents of this disease, (2) the development of sensitive, efficient and rapid assay systems by which the epidemiologic importance of the disease can be determined, (3) the in vitro cultivation of type 2 human rotavirus, (4) the ability to induce illness in human volunteers and experimental animals administered the human rotavirus, (5) the elucidation of the importance of intestinal fluid IgA rotavirus antibody in preventing rotavirus illness under experimental conditions, (6) development of biophysical and serologic methods to differentiate the human rotavirus from other related agents and (7) the elucidation of the epidemiology of the rotavirus and Norwalk viruses.

Proposed Course of Project

In future studies of the Epidemiology Section major emphasis will be given to the efficient propagation of the viral gastroenteritis agents in vitro, to the delineation of their overall importance over a sustained period in the etiology of gastroenteritis in various populations, to the immune mechanisms involved in host-defense, to the development of effective methods to prevent illness due to these agents, and to the continued search for other etiologic agents of acute infectious gastroenteritis.

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PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

STUDY OF NONBACTERIAL RESPIRATORY DISEASES IN CHILDREN

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Robert M. Chanock LID/NIAID Chief

COOPERATING UNITS (if any)
Children's Hospital National Medical Center, Washington, D.C.

LAB/BRANCH
Laboratory of Infectious Diseases

SECTION
Respiratory Viruses Section

INSTITUTE AND LOCATION
National Institute of Allergy and Infectious Diseases, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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SUMMARY OF WORK (200 words or less - underline keywords)

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LAB/BRANCH
LABORATORY OF INFECTIOUS DISEASES

SECTION
RESPIRATORY VIRUSES SECTION

INSTITUTE AND LOCATION
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SUMMARY OF WORK (200 words or less - underline keywords)

ts mutants of influenza A virus were produced and assigned to 8 recombinantion groups. Host dependent ts mutation was observed and found to be widely distributed through the viral genome. Intracistronic complementation was also observed for ts mutations affecting the P1, P2, NA and NP genes. The mutations in the ts-1A2 master donor virus were shown to affect the genes coding for the polymerase 1 (P1) and polymerase 3 (P3) proteins. Genotype analysis indicated that transfer of the P1 and P3 ts genes from the ts-1A2 donor to 11 distinct virulent, wild type viruses led to a defined and reproducible level of attenuation of these recombinants for the hamster's respitatory tract. ts-1A2 recombinants were stable genetically during growth in experimental animals. However, one doubly seronegative child shed ts+ virus late in the course of infection with a ts-1A2 recombinant. In this instance shift from the ts to ts+ phenotype was shown to be associated with an extragenic suppressor mutation.

Project Description

Production and characterization of ts-1A2 recombinant viruses - When immunity to both surface antigens of influenza A virus is lacking, only the degree of defectiveness of the vaccine virus determines attenuation and in this situation defectiveness must be greater than that specified by the ts-1[E] lesions. For this reason we constructed a recombinant (1A2) with a set of ts lesions that specified a greater degree of defectiveness than that seen with the ts-1[E] recombinants. Initially ts mutants of wild type influenza A virus were produced by chemical mutagenesis. Next, viruses with a single ts lesion were identified among the ts progeny of mutagenized virus or were produced by segregating recombinants with single lesions from mutants bearing two or more ts lesions. The single ts mutants were characterized as to complementation-recombination group and were evaluated for degree of growth restriction and genetic stability in vivo (in the hamster). In this manner we identified the 2 most defective and stable ts lesions that were genetically distinct, i.e., located on different RNA segments of the genome. Finally, we combined the 2 lesions into a single virus by genetic recombination. The resulting recombinant virus (designated 1A2) that had the A/Udorn/72 hemagglutinin and the desired two ts lesions (complementation groups 1 and 5) was more restricted in replication at 37°C than either ts parent or the ts-1[E] recombinants. Genetic evidence suggested that the 1A2 ts lesions were located on the gene coding for P3 protein and the gene coding for P1 protein; both of the affected genes are believed to code for large proteins throught to be involved in the synthesis of complementary RNA.

Genotyping of ts-1A2 recombinants - Background studies were thus undertaken to ascertain which RNA segments of the Udorn/72-ts-1A2 virus bear the ts lesions. The ts lesion in the group 1 had been shown to be on RNA segment 1 which codes for the P3 polymerase. To identify the RNA segment that bears the group 5 ts lesion the HK/68-ts-315 virus (the donor of the group 5 ts lesion to the ts-1A2 virus) was crossed with the a/WSN/1933 (HON1) wild type virus and WSN ts progeny were genotyped by polyacrylamide gel electrophoresis to determine which 315 gene co-segregated with the group 5 ts phenotype. By analyzing these WSN X ts-315 recombinants and another set of recombinants, the group 5 ts lesion was shown to co-segregate with the RNA 2 segment which codes for the P1 polymerase protein. The gel electrophoretic data thus confirm the complementation analysis which together demonstrate that the Udorn/72-ts-1A2 virus has ts lesions on its RNA 1 and RNA 2 segments which code for P3 and P1 polymerase respectively.

The acquisition of a ts gene(s) by a wild-type has been consistently associated with a reduced level of replication of the virus in hamsters' lung and with reduced virulence in man. This consistant association has led to the conclusion that ts genes are responsible for the attenuation. However, non-ts genes present in the ts donor virus can also be transferred to recombinants bearing wild type surface antigens. Since non-ts genes can also affect attenuation, it is necessary to examine the importance of these

genes as well as the ts genes in reduction of virulence. A study was therefore undertaken to determine which of the Udorn/72-ts-1A2 genes, i.e., ts or non-ts, were responsible for attenuaton. The Udorn/72-ts-1A2 virus had been mated (last year's annual report) with A/Victoria/75 wild type virus and six Vic/75-ts-1A2 recombinant viruses were isolated. One had only the P3 ts lesion and the remaining five possessed both ts lesions (i.e., ts P1 and ts P3). The single lesion virus was 100-fold reduced in its replication in the hamster's lung, whereas the Vic/75-ts recombinants that possessed both ts lesions exhibited a 10,000 fold reduction of pulmonary viral replication. The six Vic/75-ts-1A2 recombinants were genotyped to determine which gene(s) present in the Udorn/72-ts-1A2 virus conferred the property of marked restriction of pulmonary virus replication on the Vic/75-ts-1A2 recombinants. Each of the five Vic/75-ts-1A2 recombinants that received both of the ts-1A2 ts genes exhibited the same properties in vitro and in vivo and were indistinbuishable in this regard from their Udorn/72-ts-1A2 parent. The Udorn/72-ts-1A2 genes at all other loci segregated independently of these in vitro and in vivo properties. These observations indicated that the two ts-1A2 ts genes were responsible for attenuation and suggest that the acquisition of the two ts-1A2 ts lesions by a virus can effect a predictable level of restriction of replication in hamsters, and, by inference, attenuation in man.

A/Alaska/77-ts-1A2 (H3N2) recombinants - The ability of the ts genes of the Udorn/72-ts-1A2 virus to reproducibly attenuate additional influenza A wild type viruses within the H3N2 subtype was evaluated further by mating this donor virus with the A/Alaska/77 wild type virus. The four Alaska/77 ts-1A2 recombinant viruses that received the P3 and P1 ts genes of the Udorn/72-ts-1A2 virus had a 37°C shutoff temperature and exhibited restriction of growth in pulmonary and nasal turbinate tissues similar to their attenuated Udorn/72-ts-1A2 parent. Eleven additional Alaska/77-ts-1A2 recombinants were obtained and fell into two groups: (1) segregants with the group 1 (P3) lesion and (2) segregants with the group 5 (P1) ts lesion.

The relative contribution of the ts P3 and P1 genes to attenuation and genetic stability of the Udorn/72-ts-1A2 virus and that of its recombinants was estimated by evaluating shutoff temperature, level of replication in hamsters, and genetic stability of ts-1A2 single lesion segregant viruses.

The Alaska/77 ts P3 segregants were not a homogenous group of viruses with respect to shutoff temperature or level of replication in vivo. P3 segregants had a 37°, 38°, or 39°C shutoff temperature. The existence of ts P3 segregants with a different shutoff temperature allowed us to examine the varying effects of ts mutation in one gene on replication in vivo. The two ts P3 segregants with a 37°C shutoff temperature were more restricted in replication in the lungs and nasal turbinates than the 38°C ts P3 segregant. This suggests that the level of temperature sensitivity of a virus with a ts lesion in the P3 gene is a major determinant of growth restriction in vivo. These data also suggest that the defectiveness of a ts mutation, i.e.,

temperature sensitivity, at a given genetic locus specifies the extent to which replication is restricted in vivo. Lung isolates from 19 hamsters infected with ts P3 segregants retained the ts phenotype, as did 44 previously studied isolates from A/Vic/75 P3 segregants. Thus a total of 63 isolates from the lungs of hamsters infected with ts P3 segregants derived from the Udorn/72-ts-1A2 parent retained their temperature sensitivity. This demonstrates the high level of genetic stability of the ts P3 gene present in the Udorn/72-ts-1A2 parent and its recombinants.

Similar observations were made for the Alaska/77-ts-1A2 ts P1 segregants. The 38°C shutoff ts P1 segregant (clone 6) was more restricted in replication in the hamster's lungs than the 39°C (clone 33) shutoff segregant. Interestingly, and for reasons that are not clear, the 38°C ts P3 segregant (clone 249) was less restricted in pulmonary replication than the 38°C ts P1 segregant. A loss of the ts phenotype after in vivo replication was seen with the ts P1 clone 6 segregant and the Hong Kong/68-ts-315 parent. In general, the level of replication, genetic stability after in vivo replication, and shutoff temperature of the ts P1 segregants were similar to that of the Hong Kong/68-ts-315 (ts P1 donor) virus used to produce the Udorn/72 ts-1A2 virus.

The observations just described confirms that the Udorn/72-ts-1A2 donor virus has two genes with ts lesions. The ts P3 gene appears to confer on recombinants a greater degree of restriction of replication in the nasal turbinates and lungs and a greater degree of genetic stability than does the ts P1 gene. The acquisition of both ts-1A2 ts gene genes are needed to confer a predictable set of genetic and biological properties on viruses within the H3N2 subtype.

Four of the 15 Alaska/77-ts-1A2 recombinants were genotyped and it is clear that the assignment of ts genes by complementation-recombination analysis accurately identified the parental origin of the P3 and P1 ts genes in the ts-1A2 recombinants. Each of 15 Alaska/77 ts-1A2 recombinants is currently being genotyped and these genetic patterns will be compared to shutoff temperature and replication in vivo. In this way we hope to better understand the genetic mechanism(s) underlying the heterogeneity of the P3 and P1 segregant viruses.

A/Hong Kong/77 ts-1A2 (H1N1) recombinants - Transfer of the P3 and P1 ts genes present in the Udorn/72-ts-1A2 donor virus to two H3N2 wild type viruses imposed a similar level of restriction of pulmonary viral replication in the hamster on each of nine H3N2-ts-1A2 recombinants. A study was undertaken to determine if the transfer of the two ts-1A2 ts genes into a virus belonging to a different influenza A subtype, the influenza A/Hong Kong/123/77 (H1N1) wild type virus, would result in restriction of replication in vitro and in vivo comparable to that observed with the two H3N2 viruses. Eleven HK/77-ts-1A2 recombinants were obtained and these fell into three subsets. One subset had both ts lesions of the Udorn/72-ts-1A2 virus and a

37°C shutoff temperature like their ts parent. The second subset consisted of two clones with the P3 ts lesion and a 37°C shutoff temperature. The third subset contained 7 clones that possessed the P1 ts lesion and had a 37°, 38°, or 39°C shutoff temperature. When mated with each other, the HK/77-ts-1A2 recombinants, in general, interacted as expected, i.e., genetic interaction between the P3 and P1 ts segregants and a lack of interaction between viruses that shared a ts lesion. However, unexpectedly, genetic interaction occurred between one recombinant and 4 others that each possessed the P1 ts lesion. This suggested that intracistronic complementation occurred between certain P1 ts segregants. These recombinants are being genotyped to determine if they indeed derived their P1 gene from the Udorn/72 ts-1A2 parent.

The HK/77 (H1N1) wild type virus was attenuated to the anticipated level by acquisition of the two ts genes from the Udorn/72-ts-1A2 parent. The two HK/77-ts-1A2 recombinant viruses (clones 92 and 144) that received the P3 and P1 ts genes of the Udorn/72-ts-1A2 virus had a 37°C shutoff temperature. The clone 144 was examined in some detail and was found to exhibit restriction of growth in pulmonary and nasal turbinate tissues similar to that of its attenuated Udorn/72-ts-1A2 parent. All isolates obtained from the nasal turbinates of hamsters infected with parent and recombinant ts-1A2 viruses were ts. Thus, segregation of the two ts-1A2 genes into eleven separate Udorn/72 (H3N2), Vic/75 (H3N2), Alaska/77 (H3N2) and HK/77 (H1N1) ts-1A2 recombinants was regularly associated with (1) 37°C restriction of plaque formation (2), marked restriction of replication in the lungs of hamsters, and (3) a 100-fold restriction of replication in the nasal turbinates, and (4) genetic stability after replication in hamsters. Since it was demonstrated that the P3 and P1 ts genes of the ts-1A2 virus were responsible for the attenuation of the Vic/75-ts-1A2 recombinant, it is reasonable to assume that these two ts genes were responsible for the attenuation of the Alaska/77 and HK/77 ts-1A2 viruses in the present studies. Thus, the two ts-1A2 ts genes attenuated influenza A viruses belonging to two distinct subtypes to a specific and predictable level.

Characterization of ts^+ Virus Isolated from a Child Who Received Alaska/72-ts-1A2 Virus - Evidence for Suppressor Mutation - A seronegative child given a Alaska/77 ts-1A2 recombinant that had a 37°C shutoff temperature for plaque formation (clone 190) shed virus on days 7, 8, and 9 that produced plaques efficiently at 39°C. However, this altered virus failed to produce plaques at 40°C, a temperature at which wild type Alaska/77 virus produced plaques efficiently.

Among the reoviruses, which also have a segmented RNA genome, loss of the ts phenotype is usually the result of extragenic suppressor mutation (Ramig and Fields, 1979). This can be shown by backcrossing the ts^+ "revertant" virus with wild type virus and recovering ts progeny virus belonging to the original complementation - recombination group. A similar analysis was performed for the Alaska/77-ts-1A2 ts^+ isolate by mating it with

Alaska/77 wild type virus. Twenty-two percent of progeny virus from this mating was ts and each ts clone possessed the P3 ts lesion present in the ts-1A2 parent. However, the ts segregants possessing the P3 ts gene were less temperature sensitive than P3 ts segregants derived from mating Udorn/72-ts-1A2 with wild type virus; this indicates that two types of genetic alteration occurred in the Alaska/77-ts-1A2 virus during replication in a seronegative child. One type involved a suppressor mutation in another gene (i.e., not the P3 gene) that partially corrected the ts phenotype of the ts-1A2 virus. The second type was an alteration affecting the P3 and P1 genes; this type of change could result from reversion of one of several distinct mutations or it could represent intragenic suppression. A mutation probably occurred on the P3 gene of the Alaska/77-ts-1A2 ts^+ isolate because this segment migrated more slowly on polyacrylamide gel than the corresponding RNA segment of the parent Alaska/77-ts-1A2 recombinant. This observation favors the possibility that intragenic suppression was responsible for decreased temperature sensitivity of the P3 ts segregants derived from the ts^+ isolate.

The observation that the ts^+ virus was isolated from the first seronegative child who received the Alaska/77-ts-1A2 virus, whereas 77 isolates from 11 children who shed Vic/75-ts-1A2 remained ts raised the possibility that the Alaska/77-ts-1A2 virus was already genetically altered before being administered to volunteers. This was possible even though the Alaska/77-ts-1A2 recombinant had the same level of temperature sensitivity as the Vic/75-ts-1A2 recombinant. Vic/75-ts-1A2, Alaska/77-ts-1A2, and HK/77 (H1N1)-ts-1A2 viruses were therefore inoculated at a multiplicity of infection of .001 onto MDCK monolayer cultures and incubated at 34° and 37°C. Each virus caused cytopathic effects (CPE) at 34°. At 37°C none of 20 HK/77-ts-1A2, 2 of 20 Vic/75-ts-1A2 and 10 of 20 of Alaska/77 ts-1A2 virus-infected cultures developed CPE after 3 to 4 days. Only cultures infected with Alaska/77-ts-1A2 virus yielded virus at 37°C that produced plaques at 38°C. In a separate experiment each of 11 ts-1A2 recombinants (H3N2 and H1N1) containing the two ts-1A2 lesions were evaluated in the same way and only the Alaska/77-ts-1A2 virus clone 190 produced CPE at 37°C. These preliminary results indicate that the Alaska/77-ts-1A2 clone 190 virus readily changed from a 37°C to a 39°C shutoff mutant in tissue culture after 1 passage at the restrictive temperature of 37°C. This suggests that this virus underwent genetic change during recombination and/or passage and that this change increased the probability that other changes could occur (? intragenic and/or extragenic suppression) resulting in a decrease in temperature sensitivity. The simple in vitro technique used to demonstrate this phenomenon may prove useful in identifying "labile" ts-1A2 recombinants not suitable for study in man.

Production of a New ts-1[E]-like Donor Virus - It has been suggested that perhaps two donor viruses might be needed for effective immunoprophylaxis against influenza A virus. One donor, such as the ts-1A2, would be used in

(a) young children who lack experience with influenza A virus and (b) in all individuals at the time of a pandemic involving a shift in both HA and NA antigens. The other donor, similar to the ts-1[E] virus, would be used during interpandemic periods in children and adults who have some NI and HI immunity. The HK/68-ts-1[E] virus was suitably attenuated in this situation, but showed significant tendency to lose its temperature sensitivity in the hamster's lungs, in doubly seronegative children, and after passage in tissue culture, organ culture or eggs. The ts-1[E] virus has ts lesions on the P3 and NP genes and has a 38°C shutoff temperature. Segragants from the ts-1[E] virus that contained the P3 or NP ts gene had a 39°C shutoff temperature and were less stable genetically than the parental ts-1[E] virus (shutoff temperature 38°C) after replication in the hamster's lungs indicating that the ts-1[E] donor virus possessed two unstable ts genes. For this reason we produced a series of recombinant "ts-1[E]-like" viruses that contained ts P3 and NP genes that were more stable than those found in the ts-1[E] donor virus. The single lesion donors of the ts P3 and NP genes were more stable genetically than the double lesion ts-1[E] master strain. Three recombinant viruses, clone 20, 53 and 55, were isolated; these viruses possessed ts P3 and ts NP genes and had a 37°, 38°, or 39°C shutoff temperature. Current studies are directed at evaluating the genetic stability of these recombinant virus in the hamsters to determine if they are indeed more stable than the ts-1[E] virus.

Identification of a Simian Species Permissive for Influenza A Virus - Attenuation of new strains of influenza A virus can be accomplished rapidly by the transfer of attenuating genes from an attenuated donor virus to new epidemic or pandemic strains by genetic reassortment. However, evaluation of such recombinants for their usefulness in immunoprophylaxis of influenza has proceeded slowly since volunteers remain the most reliable indicator of satisfactory attenuation. For this reason it would be advantageous to identify a simian host in which recombinants could be evaluated in a preliminary manner prior to tests in man. Such a simian species would be especially useful for evaluation of host range mutants since infection of a susceptible subhuman primate species should most closely approximate that of man. The virulence of three cloned influenza A viruses for man and for three readily available species of monkeys (owl, squirrel and cebus) was compared in an attempt to identify a species of monkey that could be used to investigate the genetic basis of attenuation of influenza A viruses for man. Of the three species tested squirrel monkeys developed mild illness confined to the upper respiratory tract in response to three different viruses belonging to the H3N2 or H1N1 subtypes. This susceptibility was accompanied by a high level of virus shedding. Each of the nine squirrel monkeys that shed greater than $10^{5.0}$ $TCID_{50}$/ml of virus developed illness, whereas those that shed less remained well. None of the cebus monkeys attained this level of shedding, while 2 owl monkeys did so without evidence of illness.

Previous studies indicated that squirrel monkeys developed clinically apparent influenzal illness when given A/New Jersey/76 (Hswine N1) or an A/Aichi/68 (H3N2) virus. These findings in combination with the present observations indicate that the squirrel monkey regularly develops objective

upper respiratory illness after infection with influenza A viruses belonging to the HO-H1-Hswine or H3N2 subtypes. In contrast, the cebus monkey appears to respond less reproducibly to influenza A virus infection in that only 1 of 3 H3N2 viruses and 1 of 2 HO-H1-Hswine viruses induced illness. Thus, the squirrel monkey appears to be a moderately permissive primate host in which to investigate the genetic basis of virulence of human influenza A viruses.

Use of ELISA to Measure the Antibody Response to Influenza A and B Viruses - An important aspect in the evaluation of a live influenza A vaccine strain is determination of the human infectious dose 50 (HID_{50}). Infection with a vaccine strain is ascertained by isolation of virus from the vaccinee and/or by detection of a rise of antibody titer in the serum or nasal wash. As candidate mutant vaccine strains of greater defectiveness have been developed our ability to detect infection in vaccinees by virus isolation has decreased. Thus, increased reliance has been placed on immunological methods for detection of infection. The hemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) techniques are the most widely used methods to measure serum antibody. In some of our recent vaccine trials, we detected a serum HI antibody rise in less than 50% of vaccinees. This indicated the need to develop more sensitive tests for detection of a seroresponse to highly attenuated vaccine virus. Recently, radioimmunoassays and enzyme-linked immunosorbant assays (ELISA) were shown to be more sensitive than conventional serologic tests for detection of serum antibody to influenza virus. For this reason the simpler ELISA was adapted to detect rises in serum antibody in vaccinees who received an influenza A wild type or vaccine virus.

Pre and post-vaccination sera were diluted and assayed on plates containing whole, zonally purified, inactivated virus. The area between the pre and post immunization curves (optical density versus serum dilution) was determined. Areas greater than 50 were indicative of an antibody rise. ELISA was more sensitive for detecting an antibody response in recipients of live virus vaccine than were HI and NI tests. With H1N1 and H swine N1 viruses, ELISA was superior to NI and HI for detection of an antibody response. This was also true for the H3N2 virus tested. Of interest, an anti-human IgG conjugate was not able to detect all rises by ELISA. Only 3 of 8 H1N1 vaccinees developed a response when tested with an anti-IgG conjugate, whereas, each of the 8 vaccinees developed a response detectable with an anti-IgM conjugate. Since these vaccinees lacked prior experience with H1N1 surface antigens, the predominance of IgM antibody in their primary response was not unexpected.

Use of whole virus as antigen, in the ELISA measures antibody responses to the surface glycoproteins (the hemagglutinin and neuraminidase) as well as to the matrix protein and nucleoprotein. Antibodies to the hemagglutinin and neuraminidase are associated with resistance to influenza A virus, whereas the other antibodies do not appear to contribute to resistance. To measure only those antibodies associated with resistance, hemagglutinin and

neuraminidase specific ELISA were developed. Wells of a microtiter plate were coated with 200 ug of purified hemagglutinin from the A/USSR/77 (H1N1), A/Texas/77 (H3N2), or B/Hong Kong/72 virus produced in collaboration by Dr. Michael Phalem of BOB, FDA. Sera from vaccinees were assayed using an ELISA and HI test against homologous and heterologous hemagglutinins. Rises were detected by ELISA and HI only against homologous antigens. However, the ELISA was more sensitive in detecting rises than the HI test with each of the three hemagglutinins; an ELISA rise of 1.5 $\log_2$ occurred for each 1.0 $\log_2$ HI rise. Sera from children who had not been exposed to influenza A or B virus possessed ELISA titers of $\leq$1:20 and post-infection sera registered titers of 1:640 to 1:20,460. The ELISA therefore greatly extends the range of detection of antibody compared to the conventional HI test. There is a good correlation between HI and ELISA titers with HI titers of 1:8 corresponding to ELISA titers of 1:160 to 1:640. Modifications of the ELISA to detect neuraminidase antibody and to detect nasal wash antibody are in progress.

Genotyping of influenza A virus recombinants - New ts mutants derived by 5-FU mutagenesis of influenza A Udorn/72 virus were isolated and characterized as previously described. These were assigned to complementation groups represented by seven HK/68 prototype ts mutants and two new complementation groups. One of these mutants, Udorn/72-ts-368, was evaluated for the locus of its ts lesion by a mating with WSN/33 wild type virus. We selected for recombinant viruses that were ts (39°C titer/34°C titer 10^{-5}) and retained the WSN/33 hemagglutinin (HO). Ten such recombinants were cloned and evaluated by polyacrylamide gel electrophoresis (PAGE) in the presence of 6M Urea, as previously described. All of these viruses retained RNA 6 of the Ud/72-ts-368 parent virus, and one recombinant clone 218, was shown to have received seven RNA segments from the WSN/33 wild type parent and only one, RNA 6, from its Ud/72 ts parent. RNA 6 is known to represent the NP gene of Ud/72 virus under these conditions of electrophoresis. Clone 218 was further evaluated for efficiency of plaque formation and in complementation-recombination assays. In each instance, it behaved exactly as its Ud/72-ts-368 parent, indicating that mutation in the Ud/72 RNP gene of clone 218 accounts for all of its properties as a ts virus. These results were consistent with earlier findings by others that HK/68 ts mutant R8, our prototype complementation group 2 mutant, also bears its ts lesion in the NP gene.

Attempts were made to determine the locus or loci of the ts lesion or lesions of several other newly derived Ud/72 ts mutants, in particular those that bear ts lesions not represented among our previously existing prototypes. We were not successful in obtaining true ts recombinants (39°C titer/34°C titer 10^{-5}), when we mated these viruses with WSN/33 wild type virus. This may be due to the phenomenon of gene incompatibility. Other approaches to this problem are under consideration, such as mating the Ud/72-ts viruses with a different wild type influenza A strain as well as mating them with other ts viruses that have a lesion at a known locus and selecting for ts^+ recombinants.

Characterization of ts mutants of influenza A/Udorn/72 (H3N2)- Genetic studies of influenza A/Udorn/72 (H3N2) virus ts mutants, isolated from virus mutagenized with ICR191 (an acridine-based compound), nitrous acid or ultraviolet light, were continued. Since we noticed that temperature sensitivity of mutants varied significantly from test to test, we had to reevaluate the temperature sensitivity of each mutant in several tests. If a mutant had a plaque titer 40°/34° $\leq 10^{-3}$ in more than a half of the tests and $\leq 10^{-4}$ in at least one-fourth of the tests, the mutant was retained for further study. Using this criterion 136 ts mutants were retained for further investigation. Eighty mutants were ts on both RMK and MDCK cells, 3 were ts on only RMK cells, and 53 were not ts on RMK cells but were ts on MDCK cells.

The 83 RMK ts mutants were arranged into 13 complementation groups, groups 1 to 13, by extensive complementation assay on RMK cells at 40°C. The progeny of the crosses between prototypes of the 13 groups were examined for emergence of wild type (ts^+) recombinants to determine if each complementation group also represented a recombination group. The progeny from the pairwise crosses involving the ts mutants of groups 1 and 11, 2 and 12, 4 and 6, and 5, 7, 9 and 12 were predominantly ts^+ virus, whereas the progeny from the other crosses were predominantly ts virus. On the basis of these findings, complementation groups 1 and 11 were assigned to complementation-recombination group C, groups 2 and 12 to group F, groups 4 and 6 to group B, and groups 5, 7, 9 and 12 to group H. Complementation groups 3, 8, 10 and 13 were assigned to complementation-recombination groups A, D, E and G, respectively. The prototype of group 12 seemed to possess ts lesions on two genes corresponding to complementation-recombination groups F and H. Thus, we obtained 8 complementation-recombination groups, A to H in the order which RNA migrates during polyacrylamide gel electrophoresis; A was least mobile etc. The number of the complementation-recombination groups, 8, is in good agreement with the number of influenza A gene RNA segments.

Complementation between ts mutants which belong to the same complementation-recombination group can be explained by intracistronic complementation. Mapping the ts locus of each complementation group to its viral RNA segment provided direct evidence for occurrence of intracistronic complementation. The mapping was done by gel electrophoresis of RNA from ts^+ recombinants produced by crossing an A/Udorn/72 ts mutant and an A/WSN/33 ts mutant whose corresponding RNA segments could be distinguished by this method. Each of the independent ts^+ recombinants should have a WSN RNA segment corresponding to the Udorn RNA segment on which the ts lesion resides. Using this method complementation group 3 of complementation-recombination group A was mapped to RNA 1 which codes for P3 protein. Groups 4 and 6 belonging to group B, were mapped to RNA 2 which codes for P1 protein, although we could not exclude the possibility that group 6 has other ts lesions on RNAs 7 and 8. Groups 1 and 11 of group C, were mapped to RNA 3 which codes for P2 protein. Group 8 of group D, was mapped to RNA 4 which codes for HA protein. Group 10 of group E, was mapped to RNA 5 which codes

for NA protein. Group 2 of group F, was mapped to RNA 6 which codes for NP protein. Group 15 of group G, was mapped to RNA 7 for M protein or RNA 2 for P1 protein. Groups 5, 7 and 9 all belonging to group H, were mapped to RNA for NS protein although there is a possibility that group 5 might have additional ts lesions on RNA 3 and 6. The prototype of group 12, which is thought to have two ts lesions belonging to groups F and H, was mapped to RNAs 6 and 8. It was shown that intrasistronic complementation could occur with ts mutations that affect the gene coding for P1, P2, NP or NS protein. It was also demonstrated that each of the 8 complementation-recombination groups corresponded to one of the 8 genomic RNA segments without overlap, although the mapping of group G on RNA 7 is not yet conclusive. New prototypes of the 8 complementation-recombination groups A to H, were selected. The new prototypes were more stable than the corresponding prototypes of the original 13 complementation groups. The new prototypes also failed to complement with the prototypes of complementation groups which belonged to their respective complementation-recombination group. The progeny of pairwise crosses of these 8 mutants were examined for temperature sensitivity. Each of the crosses yielded predominantly ts^+ virus. This confirmed that the 8 new prototypes represent 8 non-overlapping complementation-recombination groups. The remainder of the mutants were crossed with the 8 prototypes to locate the ts lesions that corresponded to complementation-recombination groups A to H. Each of the remaining mutants had one or more ts lesions represented within the 8 complementation-recombination groups. This indicated that the 8 groups covered all complementation groups present in the 83 ts mutants. Sixty-one of the 83 mutants appeared to be single lesion mutants: 13 mutants were group A mutants; 16, group B; group C; 1 group D; 1 group E; 16, group F; 1 group G; and 4, group H. Twenty-one mutants had ts lesions of two groups and one mutant had ts lesions of three groups.

Some pairs of mutants that complemented on RMK cells failed to complement each other in MDCK cells. This host dependency of complementation was also seen in crosses between the 8 prototypes. Therefore, we had to choose another set of prototypes for assay on MDCK cells. The ts lesions of 133 MDCK ts mutants were assigned to the 8 complementation-recombination groups, A to H, by performing crosses with the 8 prototypes selected for assay on MDCK cells. There were 85 single lesion mutants: 22 mutants of them were group A mutants; 22, B; 17, C, 1, D; 2, E; 16, F; 2, G; and 5, H. No mutant complemented all of the 8 prototypes suggesting that there are not more than 8 complementation-recombination groups in influenza A virus.

Sixteen mutants showed distinct host dependency of temperature sensitivity. These mutants plaqued equally on MDCK and RMK cells at 34°C. However at 40°C MDCK/RMK plaque titer was $< 10^{-3}$, and plaque titer 40°/34°C on MDCK cells was $\leq 10^{-4}$ but on RMK cells was $\geq 10^{-2}$. Thus, the 16 mutants appeared to be temperature dependent, host range (td-hr) mutants. The 16 td-hr mutants did not share a common td-hr lesion but instead fell into 8 groups by complementation assay on MDCK cells at 40°C. The involvement of host-dependent suppressor mutation in this td-hr phenomenon was examined by segregation analysis in which a host independent ts marker from a td-hr

mutant (SP392) was sought during a mating with host independent ts mutants. The results indicated that a suppressor mutation was not involved in SP392 and that a host dependent ts mutation was responsible for the temperature dependent host range restriction. Although we did not do this test with all of the td-hr mutants, our results strongly suggested that each influenza gene can undergo td-hr mutation.

Seven mutants, 3 of group A, 4 of group B, 1 of group C and 2 of group F, had a shutoff temperature of 37°C or 38°C on both of RMK and MDCK cells. Therefore, these 7 mutants have potential for use as vaccine strains or as a donor of a ts lesion to construct stable multi-lesion ts recombinants.

Publications

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Murphy, B.R., Wood, F.T., Jr., Massicot, J.G., Chanock, R.M.: Temperature sensitive mutants of influenza virus. XVI. Transfer of the two ts lesions present in the Udorn/72-ts-1A2 donor virus to the Victoria/3/75 wild type virus. Virology 88: 244-251, 1978.

Grizzard, M.B., London, W.T., Sly, D.L., Murphy, B.R., James, W.D., Parnell, W.P., and Chanock, R.M.: Experimental production of respiratory tract disease in cebus monkeys following infection with influenza A/Victoria/5/75 or influenza A/New Jersey/76 virus. Infect. Immun. 21: 201-205, 1978.

Chanock, R.M.. Murphy, B.R., Spring, S.B., Markoff, L.J., Richardson, L.S., and Belshe, R.B.: The use of live mutants for the prevention of respiratory tract disease. Acta Pathologica et Microbiologica Scandinavica, in press, 1979.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00026-12 LID

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Laboratory and Epidemiologic Studies of Viral Hepatitis Agents

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

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SECTION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
13	12	4

CHECK APPROPRIATE BOX(ES)

XX (a) HUMAN SUBJECTS X (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project consists of continuing studies of the chemistry, structure, epidemiology, immunology and pathology of the human hepatitis viruses. The goal of such studies is the control of human viral hepatitis by application of the most appropriate methods, including active and passive immunization, chemotherapy and interdiction of spread of the viruses. Progress: Partial biophysical and biochemical characterization of hepatitis A virus has been achieved and studies of hepatitis type A in non-human primates, using defined pools of virus, are almost complete. There is preliminary evidence for successful cultivation of hepatitis A virus in vitro. An inactivated subunit vaccine for hepatitis type B has been developed and is undergoing extensive tests of safety and efficacy in man. A third hepatitis B antigen, e antigen, is being characterized and its relationship to infectivity is being explored. Evidence that populations of hepatitis B viruses may contain defective interfering particles has been obtained, and this finding is being utilized in renewed attempts to isolate the virus. A newly recognized clinical syndrome, type non-A, non-B hepatitis has been further defined and attempts to identify an etiologic agent intensified through transmission studies in chimpanzees.

Project Description

Objectives: (1) To collect clinical material of high potential infectivity for use in laboratory studies, (2) To develop and test in vitro procedures and animal systems for use in laboratory studies of viral hepatitis, (3) To propagate the viruses of hepatitis in culture or in experimental animals, (4) To study the biochemistry, biophysics, immunology and epidemiology of hepatitis-associated antigens; (5) To develop vaccines or other control measures for viral hepatitis; (6) To develop chemotherapeutic approaches to the control of chronic viral hepatitis.

Methods Employed

(1) Longitudinal collection of clinical specimens from hepatitis patients.

(2) Ultracentrifugal, electrophoretic and column chromatographic purification of hepatitis viruses and related antigens.

(3) Inoculation of non-human primates for the determination of infectivity and clinical spectrum of hepatitis viruses.

(4) Microscopic examination of hepatitis viruses and virus-infected tissue by light microscopy, fluorescence microscopy, and electron microscopy.

(5) A wide variety of tissue culture techniques.

(6) A wide variety of immunologic techniques for measuring humoral and cellular immune responses.

(7) Application of DNA recombinant techniques to the study of the hepatitis viruses.

Major Findings

(1) Type B Hepatitis

HBsAg

Because of its potential usefulness as the raw material for hepatitis B vaccine, HB_sAg is being intensively studied. Preparation of two pilot lots of vaccine, one subtype adw and the other subtype ayw, has been completed by Dr. J. Gerin, in a collaborative program.

Although a hepatitis B vaccine prepared from HB_sAg purified from the plasma of chronic carriers of the antigen is feasible and probably cost-effective for its contemplated uses in the United States, such a vaccine probably will not be cost-effective for the people most in need of a vaccine in the developing world. Therefore, additional studies on alternative methods of purification and inactivation, comparisons of monovalent vs. bivalent or

polyvalent vaccines, studies of different combining ratios of mononvalent vaccines into bivalent preparations, different vaccine dosages, different vaccination schedules, different routes of administration, studies of possible adjuvants and their interaction with vaccine preparations and further characterization of the immunizing antigens themselves are being carried out.

Four modified vaccines have been prepared. Two of these have been extracted with ether-tween 80; 2 are alum-precipitated. These vaccines are currently being evaluated for immunogenicity in volunteers. Preliminary results indicate that at least certain of the vaccines are much more immunogenic than any previous hepatitis B vaccine tested. Thus, they produce antibody to hepatitis B surface antigen in two to three weeks instead of two to three months. Additional testing of the best of these vaccines will be carried out in voluteers to determine optimum immunization schedules.

There have recently been several reports of antibodies to polymerized human albumin occurring during or after HBV infection. Other studies have demonstrated that this reactivity, rather than being antibody, is associated with receptor sites for polymerized albumin on the surface of HBsAg particles. Furthermore, there is a correlation between the detection (or titer) of such receptor sites and the presence of HBeAg. Thus, the presence of the receptor sites, like the presence of HBeAg, may correlate with relative infectivity. Dr. Hansson has studied these receptor sites and has developed a solid phase radioimmunoassay that is highly sensitive and specific. The assay is so sensitive that he is able to detect the presence of HBsAg associated with the receptor sites with a greater sensitivity than the best direct tests for HBsAg. The significance of these receptor sites remains obscure, but they may in some way be related to the attachment of hepatitis B virions to hepatocytes. Alternatively, they may bind albumin in vivo and, thereby make it more closely resemble "self". Indeed, removal of albumin from HBsAg of hepatitis B vaccines appears to markedly improve its ability to stimulate the production of antibodies.

Other HBV Antigens

HBcAg - Current interest in HB_cAg centers on the association between its detection in liver cell nuclei and the prognosis and relative infectivity of chronic hepatitis type B patients. A commercial test for anti-HB_c, based upon a radioimmunoassay first described by us is now available.

Recently, interest has centered on the detection of anti-HBc of the IgM class. The detection of such antibody would provide a means for diagnosis of a recent hepatitis B virus infection, and there is some evidence that prolongation of the IgM anti-HBc response may correlate with the development of chronic HBV infection. Dr. Hansson is developing a test for anti-HBc that is specific for IgM antibody; this test is being evaluated with serial serum samples from our extensive collection of experimental HBV infections in chimpanzees.

HBeAg - There has been much interest in this non-particulate hepatitis antigen because of its association with chronic hepatitis and relative infectivity of blood from HB_eAg positive type B hepatitis patients. Firm proof that HB_eAg is a hepatitis virus gene product is lacking, but circumstantial evidence strongly points in that direction. There have been reports that HB_eAg is a) on the surface of hepatitis B virions, b) an idiotypic IgG molecule and c) a modified lactic acid dehydrogenase. Probably none of these is correct: it is probably either a nonstructural viral gene product or excess viral DNA polymerase that is never assembled into complete virus. We have recently developed procedures for the purification of HB_eAg, and preparations in which host serum proteins cannot be detected have been prepared. Plasmapheresis units obtained from chimpanzees chronically infected with HBV have served as raw material for the purification.

Extensive attempts at purification and analysis of partially purified HBeAg indicate that it is a molecule of 17,000-19,000 daltons molecular weight that may occur in larger forms or as complexes with other proteins. Absolute purification has so far not been possible, and Dr. McAuliffe is attempting to produce specific antibody through the use of the mouse hybridoma system.

"Delta antigen" - This antigen was discovered several years ago by Rizzetto in Italy. It appears to be another hepatitis B antigen found, like HB_cAg, in the nucleus of infected liver cells. It has been detected only by immunofluorescence to date, but its existence has been confirmed by others. Because of the importance of thoroughly evaluating vaccine recipients for all possible markers of hepatitis B virus infection, we plan to characterize this new antigen and determine its relationship to type B hepatitis. Dr. Rizzetto is spending a year in Dr. Gerin's laboratory and LID for this purpose. Preliminary results indicate that delta antigen has a world-wide distribution, although the highest prevalence of antibody to this antigen occurs in parts of Italy. In addition, the prevalence of antibody appears to be much higher among HBsAg positive carriers who have had opportunities for multiple exposures, e.g., hemophiliacs and parenteral drug users. Seroepidemiologic data and data obtained from transmission studies in chimpanzees suggest that delta antigen may be a defective non-A, non-B agent that requires the presence of hepatitis B virus for multiplication. Attempts to discover a virion distinct from the hepatitis B virion are in progress. Activities include studying the development of delta antigen and anti-delta antigen in experimentally infected chimpanzees, determining its relationship to other hepatitis B antigens, attempting to purify the antigen from infected liver tissue (human and chimpanzee) and the preparation of reagents. This work is progressing rapidly: a radioimmunoassay for delta antigen and antibody has been developed and is being applied to seroepidemiological studies.

Hepatitis B Virus

Because HBV occurs in titers of over 10^8 infectious particles/ml in the plasma of certain chronically infected individuals, it is possible to purify these particles by large volume ultracentrifugation and characterize them. Recent collaborative studies carried out with Dr. Gerin have revealed that HB virions are heterogeneous: three populations of particles can be separated by isopynic banding in cesium chloride. The lowest density population lacks DNA polymerase activity and probably most if not all nucleic acid. The highest density population is strongly positive for polymerase activity and contains double-stranded circular DNA that can be extracted and characterized. Most interesting is the intermediate density population which, itself, is heterogeneous and which is weakly polymerase positive and contains nucleic acid with a molecular weight only 80-90% of that of the highest density particles. Thus, these particles have all of the biophysical characteristics of defective interfering particles, entities that in other classical virus systems have been shown to inhibit fully infectious virus synthesis in vitro and to modulate virus infection in vivo in certain laboratory animal model systems, converting a rapidly progressive infection to a persistent or chronic one. Such a defective interfering particle system has never been described for a human disease. This finding has important implications for the two following topics.

Isolation of HBV in vitro

Despite many attempts over many years by many investigators, HBV has never been successfully isolated and serially transmitted in vitro. The reason for this is not known and may be related to a number of factors including limited host range (HBV is known to grow only in hepatocytes of humans and certain species of apes closely related to man). However, one reason for failure to isolate the virus may be that the virus is always found in the presence of a vast excess (up to a million-fold more particles) of viral coat material (HB_sAg) as well as a variable concentration of defective interfering-like particles. The former can compete for receptor sites on sensitive cells, and the latter can inhibit the synthesis of infectious virus when they coinfect cells with intact virions. To examine this, preparations of high density and intermediate density HB virions have been purified by isopynic and rate zonal centrifugal procedures under conditions of high containment in Dr. Gerin's laboratory. These preparations are being titered for infectivity in chimpanzees; they are being used for attempts to isolate HBV in a number of tissue culture cell lines.

Coincident with these studies is the development of reagents for identifying all of the markers of HBV infection. These assays are being used in evaluating various clones of hepatocytes derived from human or chimpanzee liver. In collaboration with researchers at Columbia University, we are attempting to produce hybridoma cell lines derived from fusions of

hepatoma cells and normal hepatocytes. Hopefully the resulting cell hybrid will have the immortality of the hepatoma cells but retain the metabolic patterns and virus sensitivities of the hepatocytes. Cell lines that can be certified as being of liver origin will be inoculated with partially purified HBV that is being titered in chimpanzees. These inoculated cultures will be monitored with the various assays available in the laboratory including radioimmunoassays for HB_sAg and HB_cAg, HBV-specific DNA polymerase enzyme activity, visualization of HB_sAg and HB_cAg particles and HB virions, immunofluorescent detection of HB_sAg or HB_cAg and detection of intracellular HBV genome by nucleic acid homology techniques.

Although HBV has not been successfully cultured in vitro, recently a hepatoma cell line was isolated that synthesizes HB_sAg. This cell line, derived in South Africa, has been distributed to others, and we are conducting a detailed characterization. It is recognized that this cell line probably cannot serve as a source for hepatitis B vaccine because of its malignant origin, but it may yield important information on HBV-cell interaction. Attempts to recover the virus by cocultivation and induction with anti-metabolites have been unsuccessful to date, but data from several laboratories indicate that the cell line may contain up to three virus genomes per cell.

Although only HBsAg can be detected in this cell line, it is not known whether small quantities of infectious virus are produced. Such production would make the cell line considerably more hazardous than if infectious virus were not produced. To determine the infectivity of this cell line we have inoculated sero negative chimpanzees with harvested tissue culture fluid and with viable hepatoma cells. This experiment is still in progress.

Pathology

Although much has been learned about hepatitis B virus and its antigens, the method by which it produces disease is still poorly understood. There is some evidence that HBV is not cytopathogenic but, rather, produces tissue damage through the host's immune response to infection. Thus, it has been postulated that the host's humoral or (more probably) cellular immune response to viral antigens or modified liver cell antigens may lead to cell damage or death. Without going into detail here, there is evidence both for and against this hypothesis. However, attempts to prove the immunological nature of viral hepatitis have been unconvincing for a number of reasons, including difficulty in measuring and interpreting cellular immune responses in man. It is unlikely that this question can be adequately answered until autologous target cells infected with HBV can be maintained in vitro and lymphocytotoxicity between these cells and lymphocytes obtained prior to infection, during the acute phase of illness and during convalescence can be studied in vitro. This is not

technically feasible at present, but studies of the possible role of anti-idiotype antibodies in modulating HBV infection are feasible and will be attempted. At present attempts to demonstrate a cellular immune response in chimpanzees with traditional in vitro methods are being repeated. In these experiments lymphocytes are being harvested at weekly intervals, and skin tests with purified HBsAg are being applied.

However, there is an alternative explanation for the variable course of type B hepatitis: the infection may be modulated by the ratio of defective interfering-like particles and fully infectious virus. This hypothesis is amenable to experimental analysis. We plan to experimentally infect chimpanzees and obtain serial plasmapheresis units from the animals throughout the course of infection. These plasmapheresis units will be analyzed for the ratio of the various density populations of HB virions as a function of phase of the disease and the results correlated with analyses of serum HB_sAg and HB_eAg titers, serum liver enzyme levels, intrahepatic HB_cAg and HB_sAg and histologic evidence of liver damage. If the results of these experiments warrant it, consideration will be given to combining purified heavy density and intermediate density HB virions in different ratios and inoculating chimpanzees to determine if the clinical course of type B hepatitis in the animals can be predicted.

Meyer zum Buschenfelde produced a chronic hepatitis in rabbits by exhaustive immunization with liver tissue. He demonstrated that an antigen on the surface of hepatocytes (liver specific protein, LSP) was responsible for initiating this immunological disease. A similar LSP-anti-LSP system was shown to be present in cases of human chronic hepatitis. In independant studies and in studies in collaboration with our laboratory, Meyer zum Buschenfelde has shown that such anti-LSP-positive chronic hepatitis patients appear to have a disease distinct from hepatitis B virus-induced chronic hepatitis. However, this has not been rigorously proven, and the published concern that hepatitis B vaccine hypothetically contaminated with LSP might lead to chronic hepatitis in vaccine recipients has led us to begin studying LSP in more detail. LSP appears to be an extremely labile membrane-associated antigen that has not been purified, although one group in England believes that they have effected a purification. Earlier reports of various assays for LSP are now suspect, and the demonstration of LSP on liver cell surfaces by indirect immunofluorescence appears to be the only reliable assay method at present. We have recently established this assay in the laboratory and are beginning to study this antigen-antibody system. There is a great need for the development of more sensitive and less cumbersome tests for measuring LSP and anti-LSP and for the preparation of hyperimmune serum. If sensitive assays such as radioimmunoassays can be developed for LSP and anti-LSP, they will be applied to serial serum samples from chimpanzees and humans with type B hepatitis (and other types of hepatitis) to determine if there is evidence for this type of immunological response in viral hepatitis. The results will go a long way toward resolving the question of whether hepatitis is an immunological disease.

Antivirals and Chemotherapy of Type B Hepatitis

With the almost simultaneous reports of modification of chronic hepatitis B virus infection in humans or chimpanzees by three laboratories (ours, Merigan's at Stanford, and Desmyter's in Belgium) in 1976 a new era in type B hepatitis research began. Both interferon and an interferon inducer were shown to markedly diminish all of the markers of hepatitis B virus infection when they were administered in sufficient doses and for a sufficiently long period of time. Subsequent studies have shown that interferon is not the "magic bullet" for treatment of chronic hepatitis, and there is still insufficient information about the toxicity of certain interferon inducers to use them in large doses in humans, but the studies did show that the treatment of chronic hepatitis can be approached from the standpoint of treating the virus rather than the host. All previous treatment regimens have been concerned with suppressing the host's immune response with steroids and antimetabolites in an attempt to diminish the clinical manifestations of disease. These studies have recently been critically reviewed by the Washington, D.C., VA liver group and their conclusion was that steroids and antimetabolites have not been shown to be effective in the treatment of chronic type B hepatitis, a finding in agreement with the observations of others.

Further evidence that chemotherapy holds promise for the treatment of chronic type B hepatitis comes from recent studies by Merigan in which the antiviral drug adenine-arabinoside (Ara-A) was also shown to markedly diminish the titer of the various markers of hepatitis B virus infection in two patients with chronic type B hepatitis. However, there was significant toxicity associated with treatment with this drug.

An important aspect of the interferon/interferon-inducer studies is that a chronic virus infection was modified. In in vitro and in vivo studies of the action of interferon this substance has been found to be relatively ineffective after infection of cells has taken place: its primary mode of protection is by preventing spread of virus from infected cells to uninfected cells. This has very important implications for the understanding of chronic type B hepatitis and suggests that such chronic hepatitis B virus infection is an ongoing disease in which infected cells produce new virus, die and are replaced by new cells that are subsequently infected with virus and pass through the same cycle. Interruption of that cycle of synthesis of new virus and transmission to uninfected cells may be the key to terminating chronic type B hepatitis. This becomes even more significant in light of one of the key observations in our interferon-inducer study in chimpanzees: we were able to show a marked increase in the ratio of defective (polymerase-negative) hepatitis B virions to polymerase-positive virions as a result of treatment of the chimpanzees with interferon inducer and at a time that coincided with the fall in titer of the other markers of hepatitis B virus infection. This would be consistent with our hypothesis that chronic type B hepatitis is a defective-interfering virus system in which modulation of the

infection can be achieved by altering the ratios of infectious and defective particles. Verification of this is technically possible by the methods described above and by treating chronically infected chimpanzees with interferon, interferon inducers or other antivirals and monitoring the ratios of different hepatitis B virions by sophisticated isopycnic banding procedures.

Recently, Nordenfelt has shown that another antiviral agent, phosphonoformic acid, inhibits the DNA polymerase of HBV in vitro. We are examining the in vivo effects of phosphonoformic acid in chimpanzees. Preliminary studies indicate that PFA may exert an antiviral effect on HBV in chronically infected chimpanzees in the absence of significant toxicity. More detailed toxicity data are being obtained in treated chimpanzees, with the hope of testing this drug in patients chronically infected with HBV.

Other Studies

Although much has been learned about the epidemiology of type B hepatitis in recent years, there are several intriguing questions still unanswered. One stems from the discovery that approximately 0.1% - 0.5% of the United States population are carriers of HB_sAg; a proportion of these have associated chronic persistent hepatitis or chronic active hepatitis, the latter a life-threatening disease. What is totally unknown, however, is what proportion of the population has evidence of chronic hepatitis of any etiology. There is evidence that, as with hypertension, there is a large reservoir of undiagnosed disease not recognized because it has not been systematically sought. In collaboration with others, we are initiating a study of the Framingham population for evidence of occult chronic hepatitis. When identified, such patients will be evaluated and the etiology of their hepatitis determined. If the results of initial surveys warrant it, we would attempt long-term followup of the Framingham population in an attempt to determine the incidence and outcome of such disease by etiology.

The rapid progress made in the development and application of DNA recombinant technology to virology has very significant implications for the study of hepatitis viruses. Already, the genome of HBV has been cloned and sequenced in its entirety. In addition, the location of the genes that code for HBsAg and probably HBcAg have been determined. A cloned HBV genome is being made available to our hepatitis program. The availability of large quantities of pure HBV DNA will make possible difinitive studies of the virus and its relationship to the infected hepatocyte, as well as its relationship to hepatic cell carcinoma. Ultimately, the translation of gene products from the HBsAg gene of the HBV genome may provide an inexpensive and safe hepatitis B vaccine.

Several variants of HBV are being examined in the chimpanzee model system. Among these are strains of HBV that have been obtained from patients with Gianotti-Crosti syndrome. This disease, most common in

Italy, is a papular dermatitis that occurs in young children acutely infected with certain strains of HBV. The disease was introduced into Japan several years ago when an Italian freighter docked at a Japanese port; the disease has spread in a concentric pattern from the harbor of the city since that time. Young chimpanzees have been inoculated with two strains of HBV associated with the disease; they are being followed for evidence of the characteristic skin rash. Should it occur, it would be characterized and further characterization of the virus performed to determine the nature of these variant viruses.

Recently we have carried out seroepidemiologic studies of hepatitis B (and A) virus infection in isolated island communities of the South Pacific. Both viruses were found to be endemic in this setting. Significant differences in prevalence were observed on different islands, but these differences could not be related to island size, culture or geography. The only exception to this was the observation that hepatitis A virus infection apparently could not be sustained on a relatively small island (Ponape) where the susceptible (seronegative) proportion of the population was depleted.

(2) Hepatitis A Virus

Characterization of the virus

Although hepatitis A virus (HAV) has recently been isolated in vitro, sufficient particulate viral antigen(presumably HAV) has been available to a very few laboratories to begin biophysical and biochemical characterization. Two preliminary studies indicate that the viral particles have peptides similar to those of picornaviruses. Although definitive nucleic acid studies have not been completed, two studies suggest that the viral nucleic acid is RNA but possibly of lower molecular weight than that of picornaviruses.

Antigen(s)

Only one antigen, hepatitis A antigen (HAAg) has been associated with type A hepatitis infections to date. Four serologic techniques for detecting this antigen and antibody to it have proven most useful for serologic studies of type A hepatitis infection: these are immune electron microscopy, immune adherence hemagglutination, radioimmunoassay and enzyme-linked immunoassay. Recently we have developed an immunofluorescence assay for hepatitis A antigen that has permitted us to study the tissue distribution of viral infection in chimpanzees and marmosets. HAAg was detected in the liver, germinal centers of lymph nodes and spleen, and basement membrane of the kidney. The latter sites probably represent sequestration of antigen released from the liver into the circulation. Interestingly, viral antigen has not yet been detected in the gut.

However, only animals intravenously infected had been examined. However, we recently studied gut tissue obtained sequentially from marmosets experimentally infected with HAV via the oral route.

The small and large intestines of marmosets killed sequentially during the incubation period and acute phase of experimental hepatitis A virus infection were examined for hepatitis A viral antigen by immunofluorescence and radioimmunoassay. Although antigen could be detected by both techniques in the liver and bile after an appropriate incubation period, evidence of virus multiplication could not be found in any part of the intestine or mesenteric lymph-nodes in any of the animals. Thus, we have not been able to demonstrate an enteric phase of multiplication for the hepatitis A virus.

The development of an immunofluorescence test for hepatitis A viral antigen permitted Provost and Hilleman to identify hepatitis A virus multiplication in tissue culture. The inoculum for their tissue culture studies was a strain of HAV that had been passaged in marmosets for 31 times. We have confirmed the multiplication of hepatitis A virus in tissue culture after marmoset passage. Three strains of HAV have been serially passaged in marmosets; at least two of these (and possibly the third) produce hepatitis A viral antigen detectable by immunofluorescence in African green monkey cells. Serial passage in this cell line is currently being attempted, and the parameters that affect growth in culture (number of passages in marmosets, titer, etc.) are being examined.

As with type B hepatitis, hepatitis A virus infection leads to the development of IgM antibodies early in infection, followed by the development of IgG antibodies. Because anti-HAV appears early in the acute phase of illness, serologic confirmation of infection is difficult to obtain unless both acute phase and late convalescent phase sera are available for comparison of titer. Even then, acute phase titers are apt to be as high or higher than convalescent titers when tested by radioimmunoassay. Recently several laboratories have developed excellent radioimmunoassays for detection of IgM anti-HAV. Dr. Hansson has developed such a test in our laboratory and has evaluated its sensitivity and specificity with serial serum samples from experimentally infected chimpanzees. He has used the test to confirm that several epidemics of viral hepatitis were, indeed, caused by HAV. He is presently attempting to modify the test to permit specific detection of IgA anti-HAV. This latter test would have additional value as a diagnostic test and would provide another means of looking for evidence of intestinal multiplication of this virus: intestinal multiplication should lead to detectable secretory anti-HAV in intestinal contents.

Pathology

As with type B hepatitis, pathologic mechanisms involved in type A hepatitis are not understood. It appears that type A hepatitis rarely

if ever progresses to chronic disease. However, this assessment is based upon a limited serologic analysis of chronic hepatitis patients. It is now possible to directly examine liver biopsies from patients with chronic non-B hepatitis to determine if their hepatocytes contain HA Ag. Such studies have confirmed that HAV is not an important cause of chronic hepatitis.

(3) Non-A, Non-B, Hepatitis Viruses

Antigens

One approach to the identification of non-A, non-B hepatitis-associated antigens is the application of techniques useful in the detection and identification of HBV and HAV: immune electron microscopy, radioimmunoassays and immunofluorescence. By application of radioimmunoassay techniques, we have identified a serum antigen associated with two cases of well-characterized non-A, non-B hepatitis. The appearance and disappearance of this antigen in the serum was temporally related to the hepatitis in these two individuals, and the antigen was shown to be particulate and biophysically characterizable by ultracentrifugation procedures. However, insufficient quantities of serum were available for more detailed analysis. Very preliminary antibody surveys suggested that antibody to this antigen was widespread among humans and chimpanzees and that, if it is an antigen associated with non-A non-B hepatitis, it is not associated with the major cause of this disease. Carriers of this antigen are being sought among populations known to have a high prevalence of HB$_s$Ag carriage and HAV infection in order to collect sufficient material for large-scale purification and characterization.

Transmission of non-A non-B agents to chimpanzees

A number of unsuccessful attempts to transmit non-A non-B agents to primates were carried out in past years. However, recently two successful transmission studies were completed simultaneously, one by the Bureau of Biologics and the other by the Clinical Center Blood Bank in collaboration with our laboratory. In both studies the incubation period in chimpanzees was comparable to that for non-A non-B hepatitis in man, and both biochemical and histologic evidence of hepatitis was obtained. In our collaborative study, plasma or serum from patients with chronic non-A non-B hepatitis as well as from patients with acute infections transmitted disease to chimpanzees. Thus, proof of chronic carriage of non-A non-B viral hepatitis agents has been obtained.

In a collaborative confirmatory study, serial plasmapheresis units and serial liver biopsies were obtained from experimentally infected chimpanzees. Fluorescein-labeled convalescent serum from the original non-A non-B patients and from experimentally infected chimpanzees have been prepared and are being tested against acute phase frozen liver biopsies in an attempt to identify a viral antigen. In addition, the plasmapheresis units will be subjected to ultracentrifugal separations and studied by

radioimmunoassay and immune electron microscopy techniques in a search for viral antigens. Also, previously infected chimpanzees are being cross-challenged and pools of infectious plasma are being identified and aliquoted for subsequent infectivity titrations. There is now a very high probability that the application of the many techniques developed for the study of HBV and HAV will rapidly lead to the identification and characterization of non-A non-B agents. The obvious first approach to control of non-A non-B hepatitis will be the development of tests for identifying blood donors capable of transmitting non-A non-B agents. Several approaches should be considered: It may be possible to identify a serum antigen that can be detected in a manner similar to HBs Ag. Alternatively, it may be possible to detect an antibody associated with recent or chronic non-A, non-B infection, such as anti-HBc in HBV infections. Finally, it might be possible to develop or identify some non-serologic test that has a strong positive correlation with presence of non-A non-B agents. No matter what direction this research takes there will be a need for large quantities of viral antigen for the development of serologic tests, for the characterization of the agents and for seroepidemiologic studies. These will most likely come from large-volume purification of antigens from plasma of chronic carriers (one such known infectious carrier is already being plasmapheresed on a regular basis) or from extraction of antigens from liver tissue of experimentally infected chimpanzees in a manner similar to that used for production of hepatitis A viral antigen in marmosets.

Other

As part of our study of non-A, non-B hepatitis in chimpanzees, serial liver biopsies have been obtained. Dr. Shimizu has intensively studied biopsies by thin section electronmicroscopy and has found morphologic evidence for two types of non-A, non-B hepatitis. One produces characteristic cytoplasmic changes that consist of proliferation, thickening and duplication of endoplasmic reticulum. The structures so formed appear to be cylinders of modified membrane that enclose a tube of rough endoplasmic reticulum. Although the structures do not resemble known viruses, they do "breed true", that is, the same types of structures are seen in liver biopsies of chimpanzees that have received the same inoculum or that are part of serial transmission studies of the agents. In contrast, the other non-A, non-B agent produces predominantly nuclear changes that consist of shrinking, heterogeneous staining of the chromatin and, in some cells, clusters of intranuclear virus-like particles. These changes also "breed true". The nature of the structure is not well understood. Furthermore, the relationship to the etiologic agents may not be a direct one. Nevertheless, they provide the first means for differentiating between non-A, non-B agents. Attempts to partially purify and prepare antibodies to these structures are currently in progress.

The development of tests for antigens and antibodies associated with non-A non-B hepatitis will provide the opportunity for seroepidemiologic

studies similar to those carried out for types A and B hepatitis. These tests, plus the pools of virus that are being titered and certified for infectivity in chimpanzees will provide the means for attempts to isolate the virus(es) in vitro.

Woodchuck Virus

Summers recently reported the discovery of a virus of woodchucks that resembles HBV in many respects. Among these are presence of three morphological forms very similar to those of HBV, presence of a DNA dependent DNA polymerase enzyme activity, a genome consisting of circular double-stranded DNA with a single-stranded region, serologic cross - reactivity with the HBsAg and HBcAg of HBV and an association with acute and chronic hepatitis and hepatic cell carcinoma. The last association makes this virus of particular interest, because the woodchuck may serve as a useful animal model for hepatic carcinogenesis. Studies of the woodchuck virus are being carried out in collaboration with Dr. Gerin, who is characterizing the agent biophysically, biochemically and immunologically. A colony of woodchucks is being established and monitored for infection. Approximately 20-30% of wild-caught woodchucks have had evidence of infection with woodchuck virus, either at time of capture or during the first three months of captivity. Thus, the woodchuck virus appears to be widespread in nature. Plasma from these naturally-infected woodchucks has been used as a source of raw materials for development of reagents.

Most exciting has been the development of hepatic cell carcinoma in two animals that were chronically infected with the woodchuck virus. Preliminary studies of alpha-fetoprotein levels indicate that woodchucks, like man, develop elevated levels of alpha-fetoprotein when hepatic cell carcinoma is present.

Histopathologic evaluation of liver biopsies and autopsy material from normal woodchucks, chronically infected with the woodchuck virus and woodchucks with hepatic cell carcinoma is being performed by Professor Hans Popper.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00027-12 LID

PERIOD COVERED October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

BASIC STUDIES OF MYCOPLASMAS

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Joseph G. Tully, Ph.D.	LID, NIAID	Head, Mycoplasma Section
Other:	David L. Rose	LID, NIAID	Research Microbiologist

COOPERATING UNITS (if any) State Univ. N.Y., Stony Brook, N.Y. (Dr. David Williamson) USDA, Beltsville, Md. (Dr. R. Whitcomb); British Ministry Overseas Develop., Jamaica (Dr. Eden-Green); Univ. Fla., Fort Lauderdale, Fla. (Dr. R. McCoy); Children's Hospital, Washington, D.C.(Dr. H.Kim); BOB, FDA (Dr. Barile); Univ. Bordeaux (France) (Dr. Bove)

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SECTION
MCYOPLASMA SECTION

INSTITUTE AND LOCATION
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

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SUMMARY OF WORK (200 words or less - underline keywords)

These efforts cover both basic and applied aspects of mycoplasmas and related wall-free prokaryotes, including their neurotoxins, antigens and other biological factors involved in virulence, their immunological interrelationships, and their possible role in human disease or diseases of uncertain etiology. Current projects of interest concern the characterization and serological interrelationships of an expanding group of helical mycoplasmas (spiroplasmas) being isolated from plants and a variety of insects, especially ticks. Recent studies have confirmed that many of these new organisms possess overt pathogenicity for vertebrates (embryonated chicken eggs and suckling rats), suggesting that they may represent important pathogens for man. Further sero-epidemiological studies in man seem warranted. Recent collaborative studies on the acholeplasmas (non-sterol-requiring mycoplasmas) isolated from plant and animal sources suggest some alteration in the concept that these organisms are parasitic. The occurrence of free-living acholeplasmas in plants, some species of which had previously only been found in vertebrates, raises important questions about vector transmission and other host-parasite relationships of these organisms.

Project Description

Studies directed to various aspects of the biology and pathogenicity of helical mycoplasmas (spiroplasmas) have continued. Our characterization of these agents as the first known spiroplasmas with pathogenicity for vertebrates has opened up a number of areas for developing the possible role of these agents in human disease. Current work is directed to three broad areas, covering (1) the biological characterization of spiroplasmas, including their serological and biochemical interrelationships; (2) development of appropriate models to study their pathogenicity and host response, which includes not only small laboratory animals but primates, and (3) evaluation of various techniques (primary isolation in culture, serological, histological, etc.) to assess the possible role of spiroplasmas in acute and chronic human disease. We are also concerned with other mycoplasmas, particularly with the acholeplasmas (nonsterol-requiring mycoplasmas), since these strains have been recovered frequently from contaminated cell cultures and new and previously documented species of the genus Acholeplasma are being isolated from plants, insects, and a wide variety of free-living sources at present.

(A) Pathogenicity of spiroplasmas for vertebrates

Studies on the virulence of spiroplasmas for embryonated chicken eggs and suckling rats have been expanded with the availability of a number of new spiroplasmas from different hosts. The overall results of this testing program are suggestive of the presence of some unique virulence markers in spiroplasmas. The most pathogenic group of spiroplasmas is the suckling mouse cataract cluster, including strains SMCA, GT-48, and TP-2. These strains, for the most part, are highly pathogenic for the chick embryo, showing LD_{50} values of 1-10 organisms/egg. In a few instances (the TP-2 strain), an organism may show a decline in virulence with continued passage on artificial medium or after purification by filter-cloning techniques. The pathogenicity of these strains for suckling rats is more interesting. The classical SMCA strain always has shown rather low mortality for the suckling rat, with intracerebral challenge levels of 10^8 or 10^9 organisms/rat required for an LD_{50} dose. However, the survivors of this challenge level, and those animals receiving dosages of at least two logs less, showed a very high incidence of bilateral cataracts. In contrast, suckling rats challenged with the GT-48 strain have never been observed to have cataracts. Since the mortality rate in suckling rats receiving the GT-48 strain is much higher (usually 1 LD_{50} dose of this strain is near 10^4 organisms or less), it is thought that the absence of cataracts may have some relationship to surviving a massive intracerebral challenge of spiroplasmas. It is also possible that some toxic component of the SMCA strain is present in sufficient amounts in high challenge levels of the organism and information on soluble components of these spiroplasmas seems warranted. Finally, the most unique observations were made on the freshly-isolated TP-2 strain. Early passages of this strain had a pattern of pathogenicity very similar to that of the SMCA strain (high virulence for the chick embryo, low mortality for suckling rats, and occurrence of cataracts in

surviving rats). After purification and cultivation in artificial medium for 25 passages, this strain showed a complete reversal in this pattern of virulence. The egg LD_{50} titer dropped from $10^{9.0}$ to $10^{3.6}$ while the LD_{50} titer for rats increased from less than 10^{1} to 10^{5} organisms. The occurrence of cataracts disappeared with the increase of mortality in suckling rats. Thus, the TP-2 strain, with the observed changes in virulence pattern, offers an important model to study the mechanism for this transition in pathogenicity. Pathogenicity tests with other spiroplasmas for the two animal models outlined above have also led to some new insights regarding the importance of these organisms in induction of disease. Strains recovered from the honey bee (BC and KC strains) have been found to show increasing virulence for the chick embryo following passage on the SP-4 culture medium. When both strains were carried for 30-40 passages on this medium, the egg LD_{50} titer increased from less than 10^{2} to greater than 10^{8} (that is, the dose required to kill embryos changed from 10^{8} organisms/egg to less than 10 organisms/egg. While the honey bee spiroplasmas had been previously shown to be highly pathogenic for bees, the results reported here indicate that vertebrates are also very susceptible to infections with this organism. Although only limited pathogenicity studies have been done with the free-living flower spiroplasmas (OBMG and BNR1 strains), the early results indicate that these organisms are also pathogenic for the chick embryo. Studies with higher passage lines of these strains remains to be done. Pathogenicity tests with other groups of spiroplasmas, representing members of the Spiroplasma citri serological complex, have not demonstrated virulence for chick embryos or suckling rats.

(B) Serological tests with spiroplasmas

In early studies with spiroplasmas, it appeared that conventional mycoplasma serological tests were less suitable for these organisms, not only in assessing serological relationships among individual strains but in evaluating serological response to these organisms. As noted in the last report, we have developed a combined deformation/metabolism-inhibition (DF/MI) serological test for spiroplasmas and have now applied this test to an analysis of about twenty strains isolated from a variety of sources. The deformation test is based upon the observation that helical spiroplasmas in the presence of specific antibody become deformed. The metabolism-inhibition test, previously developed in LID by other investigators for conventional mycoplasmas, is based upon the ability of a specific antiserum to inhibit metabolism (in this case, glucose fermentation) of spiroplasmas grown in broth cultures. A number of test modifications were made for the spiroplasmas and the addition of fresh complement was required in the system. A combined DF/MI procedure has been adapted to microtiter plates and a frozen, standardized spiroplasma antigen prepared for all strains examined. The DF procedure has the advantage of rapid results (final readings in 1 hour), although it requires darkfield microscopy. The MI procedure requires no special equipment but final readings are only available after 3-7 day incubation period. The results of the serological analysis of spiroplasmas by DF/MI has clarifed a number of major questions about the interrelationships of these twenty strains recovered from a

number of sources by other investigators. Strains isolated from cactus and lettuce plants appear to be identical to isolates of Spiroplasma citri involved in citrus stubborn disease. Spiroplasmas from ticks (277F strain), honey bees (BC3 and KC3 strains), and from corn stunt disease (E-275 and Miss. strains), all appear to be members of a large S. citri complex and strains in this grouping all share a number of major serological determinants. We have assigned serogroup numbers to major members of the complex. The SMCA group (SMCA, GT-48, and TP-2 strains), the flower spiroplasm group I (OBMG and BNR1 strains), the flower spiroplasma group II (Powder puff strain), and the sex-ratio spiroplasmas from Drosophila species all appear to be serologically distinct groups of spiroplasmas. Further comparison of these spiroplasmas by the conventional mycoplasma growth inhibition technique have also shown similar results, indicating that this procedure may also be applied to serological analysis of spiroplasmas. The results of this analysis have provided data important to our continued sero-epidemiological study of spiroplasma antibody in the serum of man and other vertebrates. (Williamson, State Univ., of N.Y.; Whitcomb, USDA).

(C) Studies on acholeplasmas

We have continued our interest in the acholeplasmas (nonsterol-requiring mycoplasmas) since these organisms occur frequently in a wide variety of animal hosts and are, therefore, important contaminants of tissue culture systems. Two unclassified acholeplasmas, previously isolated from cell cultures and contaminated bovine serum, are being characterized. Serological tests, with a variety of techniques, and biochemical studies indicated that these two strains (72-043 and S2) are not related to other classifed species in the genus Acholeplasma. DNA-DNA hybridization techniques were performed on the two strains and comparisons were made between the radiolabeled DNA of these organisms and DNA obtained from a collection of organisms in the genus Acholeplasma and genus Mycoplasma. Control systems also included reciprocal DNA-DNA hybridization between A. laidlawii and A. granularum, where previous studies with less sensitive hybridization techniques (cellulose membrane procedure) had shown partial relationships (at about 34% level). The results of our study showed that the new acholeplasmas were not genetically related to any of the previously classified acholeplasmas, although they were closely related to each other (at about 80-85% level). A. laidlawii and A. granularum shared relatedness values of about 23% in reciprocal tests, confirming earlier judgement that these organisms were distinct species but distantly related. Efforts are in progress to complete the biological and serological characterization of the 72-043 and S2 strains as new species of Acholeplasma. (Aulakh and Barile, FDA).

Other areas of acholeplasma research involve two collaborative studies in which we are supplying assistance in the serological analysis of strains recovered from plant sources. In one study (Eden-Green, Jamaica), a number of acholeplasmas have been recovered from crowns of palms infected with lethal yellowing disease. We have typed these strains as A. axanthum and A. oculi. In extended studies, 35 isolations of acholeplasmas from 252 samples obtained from infected crowns (positive isolations in 13.8%)

have been made. Acholeplasmas were not isolated from 69 samples from healthy trees. Of the 35 isolations made, about 70% of the strains were A. axanthum and 30% are A. oculi. These observations are the first clear illustration that acholeplasmas might represent true saphrophytes. The results are also of interest since all previous isolations of both A. axanthum and A. oculi have been from animal tissues or serum. It was not fully established at present whether the recovered acholeplasmas were involved in the etiology of lethal yellowing disease, but the observations have relevance to the ecology of the acholeplasmas. The second collaborative study involves serological comparisons of a number of acholeplasmas (and a few sterol-requiring mycoplasmas) recovered from tropical flowering trees (McCoy, Univ. Fla.). Isolates recovered from two separate tree flowers have been found to share a number of properties of animal acholeplasmas, including ability to grow in a serum-free culture medium, fermentation of glucose, etc. The organisms have not been shown to be serologically related to any other acholeplasma or mycoplasma from animal hosts. As noted above, the occurrence of free-living acholeplasmas and mycoplasmas is unique and the addition of typing antisera for these organisms to a collection of reference sera might serve a useful epidemiological purpose.

(D) Mycoplasmas from clinical specimens

The SP-4 medium developed for isolation and cultivation of the pathogenic suckling mouse cataract spiroplasmas was found to increase the efficiency of isolation of Mycoplasma pneumoniae from human throat specimens (see last years report) This medium was also useful for cultivation of a number of other fastidious mycoplasmas. The current study was designed to recover spiroplasmas and mycoplasmas from throat specimens collected at the Children's Hospital National Medical Center, Washington, D.C. Bacterial overgrowth in the throat specimens was a major problem, contrary to observations made on throat specimens from Marine recruits, where the usual bacterial inhibitors in mycoplasma medium were sufficient to prevent contamination. The problem was resolved by the addition of 500 units/ml of polymyxin to the transport and culture medium, amounts which have been found to have no obvious effect on mycoplasmas or spiroplasmas. Studies have been made on 98 throat specimens and the M. pneumoniae isolation rate was about 10% on these samples. Another 196 specimens from throat swabs are under test now. A small number of spinal fluids have been tested in this study, without recovery of mycoplasma or spiroplasmas. (Kim, Children's Hospital).

(E) Other mycoplasma research

A number of other research and collaborative projects have been completed or are in progress within the section. These include:

(a) A collaborative study with investigators at the University of Bordeaux, France on the use of two dimensional slab gel electrophoresis to determine the interrelationships of spiroplasmas recovered from various sources. The results show this technique, which is essentially a finger-print of radiolabeled proteins of the individual spiroplasma, to be an effective procedure to distinguish specific spiroplasma groups.

(b) A service type study, performed on a part-time basis, to assess the occurrence of mycoplasma contamination of cell cultures (mostly continuous cell lines) in NIAID laboratories. This testing program covers culture procedures and two indirect staining methods (DNA and specific immunofluorescence tests with anti-M. hyorhinis conjugate) on indicator cell lines (Vero cells). Eighty-seven tissue culture specimens (from ten principal investigators) have been examined from October 1978 to June 1979, with 32 of the specimens being positive for one or more mycoplasmas (positive rate of 36%). This rate is not necessarily the true incidence of mycoplasma contamination, since a number of known contaminated cell lines were examined at various passage levels so that the investigator could obtain a clean cell line. However, two points should be emphasized: 1) mycoplasma detection procedure must involve the use of indicator cells since some mycoplasmas (M. hyorhinis) can lose the ability to grow on artificial culture medium and these strains can only be detected by the FA procedure, and 2) mycoplasma contamination will compromise the results of any study performed on infected cell cultures.

(c) The taxonomic characterization of the SMCA group. The results of the serological analysis of spiroplasmas (noted above) has confirmed the unique and distinct status of the SMCA group of spiroplasmas. Data on the biological and morphological features of this group of spiroplasmas can now be applied to a full characterization of the organisms (Whitcomb, USDA).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00028-21 LID

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TITLE OF PROJECT (80 characters or less)
STUDY OF RESPIRATORY VIRUSES AND MYCOPLASMAS IN VOLUNTEERS

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Brian R. Murphy, M.D.	Medical Officer	LID, NIAID
	Robert M. Chanock, M.D.	Chief	LID, NIAID
	Lewis J. Markoff, M.D.	Medical Officer	LID, NIAID

COOPERATING UNITS (if any)
Flow Laboratories, Rockville, Md.; University of Maryland School of Medicine, Baltimore, Md.; Children's Hospital National Medical Center, Washington, D.C.; University of Rochester, New York; Vanderbilt University, Nashville, Tenn.; Baylor College of Medicine, Houston, Texas

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INSTITUTE AND LOCATION
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES, BETHESDA, MARYLAND

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The response of adult volunteers to a series of ts or cold-adapted recombinants. was evaluated. The A/Alaska/77-ts-1A2 (H3N2) and A/HK/123/77-ts-1A2 (HINI) viruses, each of which had a 37°C shutoff temperature, were satisfactorily attenuated and induced an immunological response in greater than 84% of the volunteers. Virus shed by each of the volunteers retained the ts phenotype. A/Alaska/77 (H3N2) cold-adapted recombinant virus which possessed a 39°C shutoff temperature for plaque formation was also suitably attenuated and immunogenic in volunteers. The A/HK/77 cold-adapted recombinant possessed a 37°C shutoff temperature and was satisfactorily attenuated and immunogenic in volunteers. The virus shed by volunteers retained the ts and cold-adapted properties. These studies indicate that the two master strains of influenza A virus can confer, via the mechanism of gene transfer a satisfactory level of attenuation, immunogenicity, and genetic stability on virulent viruses belonging to two influenza A subtypes.

PHS-6040
(Rev. 10-76)

Project Description

Evaluation of Cold-Adapted Influenza A Virus Recombinants in Adults - Recombinant viruses produced by mating the A/AA/6/60 cold-adapted donor virus and cloned wild type A/Alaska/77 (H3N2) or A/Hong Kong/123/77 (H1N1) viruses were evaluated in adults for reactogenicity and immunogenicity. The Alaska/77 CR-29 clone 2 virus had a 39°C shutoff temperature for plaque formation and the HK/77-CR-35 recombinant had a 37°C shutoff temperature. This was so despite the fact that both viruses received all six non-glycoprotein genes (i.e., P1, P2, P3, NP, M, and MS) from the AA/6/60 donor virus. The basis for and significance of this difference in shutoff temperatures remains unknown.

The CR-29 virus (H3N2) was administered at a dose of $10^{7.5}$ $TCID_{50}$ to 24 seronegative adults (all with pre-inoculation serum HI antibody titer of $\leq$1:8 and 19 of 24 with NI titer of $\leq$1:4). Eleven shed virus in a titer ranging from $10^{0.7}$ to $10^{4.7}$ $TCID_{50}$ per ml of nasopharyngeal wash fluid for an average duration of 0.71 days which is significantly less than the interval of 4.5 days observed for volunteers who received wild type virus. Each of the 17 isolates retained the ts phenotype. Twelve volunteers developed a rise in serum HI antibody and 11 had a rise in serum NI antibody. Sixteen volunteers had a rise of HI and/or NI antibody. Two volunteers became ill; one was febrile and the other had a cold with pharyngitis and systemic symptoms. The virus was clearly more attenuated than wild type virus which (a) produced illness in 50% of the volunteers at a dose of $10^{4.2}$ $TCID_{50}$ and (b) was shed longer and in larger quantity.

The CR-35 (H1N1) recombinant was administered at a dose of $10^{7.5}$, $10^{7.0}$, $10^{6.0}$, or $10^{5.0}$ to 24, 5, 11, and 10 doubly seronegative volunteers, respectively. These studies were done in collaboration with the University of Maryland (Dr. Levine), University of Rochester (Dr. Douglas) and Baylor University (Dr. Couch). The human infectious dose 50 for the virus was approximately $10^{5.0}$ $TCID_{50}$. At doses of $10^{7.0}$ or higher 96 to 100% of the volunteers were infected. At a dose of $10^{7.5}$ $TCID_{50}$, 13% of 24 volunteers had mild, afebrile upper respiratory tract illness in contrast to an 83% incidence of influenzal illness in volunteers who received wild type virus. Illness was not observed at lower doeses. Sixty percent of volunteers who received $10^{7.0}$ or $10^{7.5}$ $TCID_{50}$ shed virus. Each virus isolate retained the ts and cold-adapted (ca) phenotypes. The shutoff temperature for plaque formation of isolates from 6 volunteers who shed virus for 6 to 9 days was determined. Of 33 isolates, 25 had a shutoff temperature similar to the parental virus, 7 were 38°C shutoff temperature mutants, and 2 were 39°C mutants. These results indicate the high degree of genetic stability of this recombinant. However, it should be noted that some genetic drift (37°C shutoff ---> 39°C shutoff) occurred. At doses of 100 HID_{50} or higher greater than 96% of volunteers had an immunological response. Thus the CR-35 virus was satifactorily attenuated and immunogenic at doses in the range of 100 HID_{50}'s.

Evaluation of ts-1A2 Influenza A Recombinants in Adults - An influenza A virus recombinant bearing the surface antigens of the A/Alaska/6/77 (H3N2) wild type virus and the two ts genes of the A/Udorn/72-ts-1A2 (H3N2) virus was evaluated for attenuation, antigenicity, and transmissibility in 28 adults volunteers all of whom had a preinoculation serum hemagglutination-inhibiting (HI) antibody titer of $\leq$1:8 and 18 of whom also had a serum neuraminidase-inhibiting (NI) antibody titer of $\leq$1:4. The Alaska/77-ts-1A2 recombinant, which had a 37°C shutoff temperature for plaque formation and ts mutations on the genes coding for the P1 and P3 polymerase proteins, infected 86% of the vaccinees when administered at a dose of $10^{6.5}$ $TCID_{50}$. Only 3% of the vaccinees developed symptoms in contrast to 50% of volunteers who received $10^{4.2}$ $TCID_{50}$ of wild type virus. Vaccinees shed virus for a shorter interval and at a lower titer than the volunteers who received wild type virus. Each ts-1A2 isolate retained the ts phenotype indicating that the recombinant was stable genetically in seronegative adults. An immunological response, as measured by a rise in serum HI and/or NI antibody, was detected in 71% of the vaccinees and 87% of the recipients of wild type virus. Transmission of vaccine virus to susceptible contacts was not observed. The two ts-1A2 ts genes have now been transferred to two variants within the H3N2 subtype, the Vic/75 and Alaska/77 viruses, and have rendered the resulting recombinants satisfactorily attenuated for seronegative adults.

The Alaska/77-ts-1A2 virus was given to children by Drs. Kim and Parrott at the Children's National Medical Center in Washington, D.C. The virus was administered to seropositive children who behaved like the adults mentioned above. One seronegative child was also studied. This individual shed virus for 10 days. Virus shed on the 7th, 8th and 9th day produced plaques at 39°C, a temperature restrictive for the ts-1A2 recombinant administered. On day 2, virus present in the nasopharyngeal wash fluid had a 37°C shutoff temperature; on day 3, 38°C shutoff temperature; and on days 7, 8, and 9 the virus had a 40°C shutoff temperature. This indicated that the A/Alaska/77 ts-1A2 recombinant gradually lost a considerable amount of its temperature sensitivity during the later period of replication in vivo. A characterization of these isolates and the role of suppressor mutation in loss of the ts phenotype are described in Project No. 28-18.

The Udorn/72-ts-1A2 virus was mated with the A/Hong Kong/123/77 (H1N1) wild type virus and a HK/77-ts-1A2 recombinant (clone 144) was isolated which had a 37°C shutoff and the two ts-1A2 ts lesions. Doubly seronegative adults (i.e., no detectable HI or NI serum antibody) were administered $10^{6.5}$, $10^{6.0}$ or $10^{5.0}$ $TCID_{50}$ of HK/77-ts-1A2 vaccine virus or $10^{4.2}$ of wild type virus. The 50% human infectious dose of the vaccine virus was approximately $10^{5.0}$ $TCID_{50}$. It was necessary to use ELISA to detect an immunological response in the vaccinees (see Project 24-18) and to accurately estimate the HID_{50}. Of 6 volunteers infected with wild type virus 5 developed febrile or systemic illness and shed virus for 5.8 days with a peak mean titer of $10^{6.3}$ $TCID_{50}$/ml of nasal wash. Eighty-six percent of 23

volunteers who received 30 HID_{50}'s ($10^{6.5}TCID_{50}$) of the ts-1A2 recombinant became infected. Illness developed in 3 volunteers, 1 febrile illness (100.2°F) of 12 hours duration and 2 mild upper respiratory tract illnesses. Only 25% of the vaccinees shed virus with an average duration of 1.7 days and peak mean titer of $10^{1.0}TCID_{50}$. Each of the virus isolates recovered retained the ts phenotype. These results indicate that the HK/77-ts-1A2 virus was satisfactorily attenuated, immunogenic, and genetically stable in doubly seronegative volunteers. Preliminary results from the challenge of HK/77-ts-1A2 and CR-35 vaccinees with wild type virus ((performed by Dr. Douglas at the University of Rochester) indicates that both vaccines induce significant resistance to infection and illness.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00175-02 LID

PERIOD COVERED

OCTOBER 1, 1978 TO SEPTEMBER 30, 1979

TITLE OF PROJECT (80 characters or less)

LABORATORY STUDIES OF PARAMYXOVIRUSES AND RESPIRATORY SYNCYTIAL VIRUS

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	Robert M. Chanock, M.D.	Chief	LID, NIAID
	Gregory A. Prince, Ph.D.	Sr. Staff Fellow	LID, NIAID
	Stephen C. Suffin, M D.	I.P.A.	LID, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

LABORATORY OF INFECTIOUS DISEASES

SECTION

RESPIRATORY VIRUSES SECTION

INSTITUTE AND LOCATION

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES, BETHESDA, MARYLAND

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
66/12	30/12	36/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Variation in level of growth of RS virus was detected for 20 strains of inbred mice tested. The difference in level of replication between the most permissive and restrictive strain of mice was sufficiently large that it should now be possible to analyze genetic control of viral replication by the appropriate cross-breeding techniques. ts-2, ts-1 NG-1 and ts-1 NG-16 mutants were shown to be highly defective and attenuated when studied in primates suggesting that these mutants may prove useful in provention of human RS virus disease. IM inoculation of virus appears to initiate an aborative cycle of replication locally leading to an immunologic response. Circulating antibody acts to suppress this response. Development of a glucose oxidase linked antibody staining technique offers the possibility of examining paraffin embedded, formalin fixed tissues for presence of RS antigens. In this manner an immunopathoarcheologic survey of RS virus and other viral pathogens can be performed using autopsy material collected previously.

PHS-6040
(Rev. 10-76)

Project Description

This year the respiratory syncytial virus program gave special emphasis to 7 areas of research: 1) genetic studies of the virus, utilizing temperature sensitive mutants; 2) continued development of animal models of RS virus infection in which to study patterns of viral growth and pathogenesis of disease; 3) in vivo evaluation in experimental animals of ts mutants and cold-adapted mutants that appeared to be promising vaccine candidates; 4) in vivo evaluation in experimental animals of bovine respiratory syncytial virus as a potential vaccine candidate; 5) immunological studies of RS virus infection in experimental animals; 6) studies of RS virus infection in the immunosuppressed host; 7) development of new or improved diagnostic techniques for the study of RS virus infection in humans and experimental animals.

Major Findings

Genetics of Respiratory Syncytial Virus

Background - RS virus is the major etiologic agent of bronchiolitis and pneumonia of early life, and the need for effective immunoprophylaxis has been clearly established in studies performed throughout the world. This virus has a non-segmented genome and does not undergo genetic reassortment. Initially a series of 7 ts mutants of RS virus were produced by chemical mutagenesis with the expectation that their temperature sensitivity would restrict growth in the target organ, the lungs.

Complementation Analysis of Additional ts Mutants of RS Virus - The complementation studies reported last year were continued and the Glasgow mutants have been further characterized. As stated previously, at least 6-8 complementation groups are likely to exist, based upon the probable molecular weight of the RS virus genome and the existence of from six to eight viral specified polypeptides. Using six prototype ts mutants isolated by Dr. Craig Pringle and his collaborators (Glasgow, Scotland), and three prototypes isolated in this laboratory, we have defined six complementation groups. The three NIH mutants represent groups we have designated Groups A, B, and C; three Glasgow mutants represent Groups D, E, and F. The remaining three Glasgow mutants have been characterized by our laboratory as belonging to Group A. Experiments varying adsorption period and incubation times showed complementation between the two mutants we had placed in Group E last year, thus establishing Group F by our method this year.

Isolation of New ts Mutants Additional ts mutants are being isolated and characterized in this laboratory in a continuing search for new complementation groups and possible vaccine candidiates. Mutagenization of wild type RS virus strain A2 (F-059, Flow Laboratories) was carried out using 5-fluorouracil and 5-azocytidine in the growth medium. Of 412 clones screened for temperature sensitivity during this year, 18 were ts. These mutants are now being characterized in vitro by complementation analysis, temperature shift

experiments to determine the time at which their ts lesions are expressed, and growth at subrestrictive temperature to determine their genetic stability. Potential vaccine candidates will then be tested in vivo, initially in the cotton rat, then in primates.

After extensive characterization of new mutants from Glasgow and NIH, none was shown to share NIH Group B (ts2 prototype) ts lesion which is characterized by non-syncytial plaque morphology and a defect in adsorption-penetration of the host cell. However, one new NIH mutant, ts111, despite its syncytial plaque morphology and early genetic lesion similar to Group C, appears to belong to a new complementation group. Additional in vitro tests currently in progress will allow us to identify it definitively.

Animal Models of Respiratory Syncytial Virus Infection

Owl Monkey - Pilot studies during the preceding year indicated that RS virus infection of owl monkeys (Aotis sp.) produced a disease characterized by rhinorrhea. Further evaluation of RS virus infection in owl monkeys was undertaken this year. Intranasal inoculation of wild type RS virus produced upper respiratory tract disease in each of seven animals. Virus was recovered from nasopharyngeal swab specimens for a mean period of 11 days, reaching a peak titer (geometric mean) of $10^{3.0}$ plaque-forming units per milliliter. Serum neutralizing antibody to RS virus began to appear 14 days after inoculation, reaching peak titer by 28 days. A combined disease score was calculated for each animal, which formed the basis for subsequent analysis of the virulence of ts mutants in owl monkeys. Because of the extreme scarcity of chimpanzees, the only other experimental animal known to develop clinically evident disease from RS virus, the owl monkey has become the model of choice for experimental study of virulence.

Inbred Mice - Five animal models of RS virus infection have been described by this laboratory, including the chimpanzee, cebus monkey, owl monkey, ferret and cotton rat. None of these species, however, is inbred, thus precluding genetic manipulation and certain types of immunologic study. Furthermore, few, if any specific immunologic reagents are available to allow study of certain aspects of RS virus pathogenesis and virus-host immunologic interaction. In an effort to develop a model for experimental RS virus infection in an inbred species, we examined twenty strains of mice. Intranasal inoculation of RS virus in infant mice produced infection in each strain examined. However, there was wide variation among the inbred strains in the amount of virus recovered from the nose and lungs. The most resistant strain, CBA/CaHN, yielded only one-hundredth the quantity of virus recovered from the most permissive strain, DBA/2N. This difference was seen in comparisons of nasal and pulmonary tissues from the two strains. Ordering of geometric mean nasal and pulmonary titers from the twenty strains demonstrated a pattern of gradual, incremental increase from relatively resistant to relatively permissive strains.

If level of viral replication were controlled by a single gene with few alleles, one would not expect to observe such a shallow linear array of

titers. This suggests that response to RS virus infection is determined by a combination of genes, or perhaps a single gene with multiple alleles. The possibility that the H-2 haplotype might have an association with resistance or permissiveness was examined by evaluating the haplotype of each strain. No connection between H-2 haplotype and extent of RS virus infection was evident, however.

The nature of the genetic factors governing susceptibility to RS virus infection in mice is not known. Our preliminary survey suggests that multiple factors are involved, but does not distinguish between control by several genes or multiple allelic control within a single gene. Since there was no overlap between the virus titers observed for the strains that exhibited the lowest and highest levels of viral growth it should be possible to analyze the genetic control of viral replication using the appropriate crossbreeding techniques.

In addition to the opportunity to study genetic control of viral replication, the availability of an inbred animal model for RS virus infection offers possibilities for studies which were not feasible previously. For instance, in vivo experiments using adoptive transfer of immunologic components, including immune cells, can now be performed. Recent experiments using immune cell transfer in mice have demonstrated the importance of cellular immunity in recovery from influenza virus infection. The similarities between influenza and RS viruses suggest that cellular immunity may also serve such a function for the latter virus.

Another advantage of the mouse model, in comparison with other models of RS virus infection, is the existence of a large number of specific immunological reagents. These include antisera directed against mouse immunoglobulins (with heavy- or light-chain as well as whole molecule specificity), interferon, theta antigen, Ly 1, 2 and 3 T cell antigens, specific histocompatibility antigens and macrophages. Such reagents should allow the eventual definition of those components of the immune system responsible for recovery from RS virus infection, as well as immunity to subsequent infection.

Finally, the existence in certain inbred mice of well-defined immunologic defects gives this model unique advantages. Animals lacking T-cell dependent, B-cell dependent, and both T- and B-cell dependent immune function have been described. Efforts to introduce such specific defects into the mouse strains most susceptible to RS virus infection are currently in progress, in cooperation with Dr. Carl Hanson, Veterinary Resources Branch, Division of Research Services, NIH. Elucidation of the role of these immunologic components in recovery from and immunity to infection would greatly increase our understanding of disease caused by RS virus.

Cotton Rat - Last year's report described the pathogenesis of RS virus infection in cotton rats. Since that time, the cotton rat has become the major animal model used in this laboratory. Despite the potential advantages of the inbred mouse model, the cotton rat will remain a major tool for RS

virus research, since it is approximately one hundred fold more permissive for RS virus than the most permissive mouse strain. Furthermore, as noted in last year's report, the cotton rat remains uniformly susceptible to both nasal and pulmonary infection throughout its life. Preliminary studies in in suggest that adult mice may be as much as one hundred fold less permissive than infants of the same strain. Therefore, we have initiated efforts to develop an inbred cotton rat, in cooperation with Dr. Carl Hanson of NIH. The inbreeding program currently is in the third generation of brother-sister mating, and will continue until an inbred line has been achieved (probably 4-5 years).

In Vivo Evaluation of Mutant Viruses

Cotton Rat Nasal Histopathology as a Marker of Virulence - As described earlier, the owl monkey is now used by this laboratory for evaluating virulence of vaccine candidates. However, the expense and relative scarcity of this primate preclude its use for routine screening of the many potential vaccine mutants being isolated. Therefore, we have examined the cotton rat as a potential model for such virulence testing. Histopathologic examination of nasal tissues from cotton rats inoculated with wild type RS virus, as described in last year's report, demonstrated a moderately severe, focal rhinitis. This finding was confirmed in more extensive studies this year, both with the Long and A-2 strains of RS virus. Furthermore, the histopathologic response of cotton rats to the ts mutants tested correlated with the response of chimpanzees to the same mutants. Specifically, the ts-1 and ts 7 mutants, which had caused rhinorrhea in chimpanzees, produced nasal lesions in cotton rats. Furthermore, the ts-2 mutant and wild type bovine RS virus, neither of which produced clinical signs of illness in chimpanzees, failed to produce lesions in cotton rat nasal tissues. We thus envision a protocol for virulence testing which would progress from cotton rats to owl monkeys and, ultimately, man.

Further in vivo Evaluation of ts-2 - Two seronegative chimpanzees were inoculated with a safety-tested ts-2 vaccine prepared by Flow Laboratories. This vaccine is currently being used in initial human trials. One animal was successfully infected in two attempts but did not develop any signs of illness, despite shedding a moderate amount of virus ($10^{4.2}$ pfu/ml of swab fluid) from the upper respiratory tract. The unavailability of further seronegative animals precluded additional study.

The same lot of ts-2 vaccine ($F_7$512) was evaluated in cotton rats. Animals inoculated intranasally with 10^4 pfu were examined daily for evidence of viral replication in nose and lungs. Of six animals initially screened (twelve tissue specimens), a single tissue yielded virus at a titer of less than 10 pfu per gram, indicating that ts-2 is minimally infective in this animal, as was observed in the chimpanzee. However, when cotton rats inoculated intranasally with ts-2 were subsequently challenged intranasally with wild type RS virus, a protective effect of the vaccine was seen, despite its apparently low infectivity. Of 21 animals inoculated with ts-2, six were

completely protected against pulmonary infection by wild type virus. Furthermore, comparision of growth of virus in the lungs of vaccinated and control animals showed a highly significant level of protection ($p < .001$). However, the level of viral replication in the nose of the two groups of animals showed no significant difference. A third group of animals, inoculated intranasally with ts-2, then reinoculated by the same route two weeks later, exhibited no further resistance than the group that received a single inoculation of ts-2.

These observations provide the first experimental evidence that ts-2 is effective in protecting against pulmonary infection by wild type RS virus. Furthermore, they suggest that the nose and the lungs may be regarded as immunologically separate, independent organs with regard to RS virus infection. Hence, failure to detect resistance in the upper respiratory tract can not be extrapolated to indicate lack of resistance in the lungs.

A final area of in vivo evaluation of ts-2 involves evaluation of temperature-competent (ts^+) progeny of ts-2. We previously reported that some isolates of virus from chimpanzees infected with high doses of ts-2 differed from the input virus in their ability to form syncytial plaques at a temperature restrictive for plaque formation by ts-2. The properties of these ts^+ viruses, especially virulence, thus assume considerable importance as regards the safety and potential usefulness of the ts-2 mutant in immunoprophylaxis. Using the cotton rat, temperature-competent viruses isolated from two chimpanzees inoculated with ts-2 were examined to determine their infectivity, pathogenicity and immunogenicity. Both "revertant" viruses were as infectious for this host as wild type RS virus. Furthermore, both were immunogenic, and protected cotton rats from subsequent challenge by wild type RS virus. However, one of the two, despite its similarity to wild type virus, failed to induce histopathology in the cotton rat's nose. This virus, previously inoculated into two chimpanzees, also had failed to produce disease. This suggests that temperature-competent progeny of ts-2 virus, are not necessaruly virulent.

The second "revertant" virus, however, did cause histopathologic changes in cotton rat nasal tissues. This virus, nevertheless, was recovered from a chimpanzee inoculated with 100-fold more virus than given the other chimpanzee. When inoculated into a seronegative chimpanzee, this second "revertant" caused rhinitis, again demonstrating the correlation between disease in primates and histopathology in cotton rats. Since current human trials employ a viral dose approximately one hundredfold lower than that given the chimpanzee that yielded the virulent "revertant", shift to a ts^+ phenotype may not be a problem when the mutant is studied in children. It is likely the cotton rat will prove helpful in evaluating this phenomenon during vaccine trials in man.

In vivo Evaluation of ts-1 NG-1 and ts-1 NG-16 Mutants - The first ts mutant of RS virus to be tested in man, ts-1, appeared promising when evaluated in adult volunteers. However, tests in seronegative infants showed the virus to possess a low level of residual virulence, and to exhibit some genetic instability. In an attempt to further attenuate ts-1, which, unlike ts-2, appears to be nearly as infective as wild type virus, nitrosoguanidine (NG) was used to remutagenize the ts-1 mutant. The two mutants evaluated in this study, ts-1 NG-1 and ts-1 NG-16, were recovered from the progeny of NG treated ts-1 and were shown to exhibit greater temperature-sensitivity and genetic stability than ts-1. We used the owl monkey to test these two mutants in vivo because it is the only experimental animal which develops clinically evident disease when infected with wild type RS virus and which is also available in sufficient numbers to permit statistically valid comparison with wild type virus.

In evaluating ts-1 NG-1 and ts-1 NG-16 in vivo, three phenomena were examined. Pattern of infection was assessed by examining the time of onset of shedding of infectious virus, duration of shedding, peak viral titer and time of peak titer. Virulence was evaluated by a composite disease score, consisting of the sum of daily disease scores for each animal. Finally, antigenicity was evaluated in terms of the time of onset and the peak titer of serum neutralizing antibody. Neither ts-1 NG-1 nor ts-1 NG-16 differed significantly from wild type virus in either duration of infection or peak virus titer. This would suggest that both mutants produced an infection that was comparable in extent to that of wild type virus, a desirable property for a vaccine strain. However, the time of onset of virus shedding and the time of peak titer of both mutants was significantly delayed compared to wild type virus suggesting that both mutants were, nonetheless, functionally defective.

Though capable of producing extensive infection both mutants were significantly attenuated compared to wild type virus. That is, the composite disease scores were significantly lower for both ts-1 NG-1 and ts-1 NG-16 infected animals. Reduced virulence, like high infectivity, is another property desirable for a potential vaccine strain. Finally, both mutants were nearly as antigenic as wild type virus, when various aspects of the humoral antibody response were measured. Again, antigenicity equivalent to that produced by wild type virus is desirable for a vaccine candidate.

These observations, in conjunction with previous studies showing ts-1 NG-1 and ts-1 NG-16 to be more defective than the ts-1, parental mutant, and more genetically stable both in vitro and in vivo, suggest that they are potential candidates for use in a live vaccine. The fact that the two mutants did not differ significantly from each other in any of the observed parameters suggests that both should be subjected to additional in vivo testing in primates and, ultimately, man.

Cold-adapted ts-1 in a Chimpanzee - In a different approach to further attenuation of ts-1 mutant, the virus was passaged 28 times at 24°C, then inoculated intranasally into a seronegative chimpanzee. This animal developed

extensive rhinorrhea and shed virus for an extended period of time, indicating that low temperature passaged virus was as virulent as its ts-1 parent.

Bovine Respiratory Syncytial Virus

Background - In 1971 a strain of RS virus, antigenically related but genetically distinct from human RS virus, was isolated from cattle in Europe. This agent, known as bovine respiratory syncytial (BRS) virus, was subsequently identified in bovine herds throughout the world. The successful use of vaccinia virus, a naturally occurring bovine virus, in the immunoprophylaxis of smallpox raised the possibility of using BRS virus as a naturally occurring vaccine against its human counterpart.

Chimpanzees - Two chimpanzees were each inoculated intranasally with BRS virus. Only one shed BRS virus. The quantity of virus was small (less than 10 pfu/ml of nasopharyngeal swab specimen) and was detected only in roller tube cultures. Six weeks later the two animals were challenged intranasally with human RS virus. Neither differed from human RS virus-inoculated control chimpanzees either in peak viral titer or extent of disease.

Owl Monkeys - Six of 9 monkeys were infected with BRS; three shed a small quantity of virus and three developed a low level rise in serum neutralizing antibody. These monkeys did not exhibit resistance when challenged subsequently with human RS virus.

Cotton Rats - One group of animals was inoculated intranasally (i.n.); the second received virus intramuscularly (i.m.); and the third was inoculated i.m. twice (2 week intervals). BRS virus was recovered from the nose and lungs of each of three animals from the first group, but at a low titer (maximum $10^{2.8}$ pfu/gm). Animals receiving BRS virus i.n. showed the most consistent antibody response, with 84% developing serum neutralizing antibody against human RS virus. Animals that received BRS virus i.m. showed a less consistent response.

When challenged intranasally with human RS virus, the three groups of cotton rats previously inoculated with BRS virus showed significant protection when compared to control animals. The greatest protection was induced by IN inoculation of BRS. The mechanism by which BRS virus stimulates immunity against human RS virus in cotton rats was not clear. It did appear, however, that serum neutralizing antibody was not responsible. Although many animals inoculated with BRS virus failed to develop detectable antibody, every animal, regardless of the route of administration of BRS virus, exhibited some resistance to subsequent human RS virus challenge. In several instances, complete pulmonary immunity was seen in animals lacking detectable antibody, suggesting that another portion of the immune system was the effector of resistance.

In spite of these promising findings in the cotton rats, the results of experiments in primates were disappointing. BRS virus may be more infectious for cotton rats, and not capable of attaining a level of replication in primates sufficient to induce an effective immune response.

Studies involving tissue culture suggest that primates are relatively non-permissive hosts for BRS virus infection. Infected bovine turbinate and bovine embryonic kidney cells each yielded from $10^{5.0}$ to $10^{6.0}$ pfu of BRS virus per ml. In contrast, human embryonic kidney, human diploid lung fibroblast and African green monkey kidney cells were relatively or completely resistant to BRS virus.

Immunological Studies in Animal Models

Maternal-to-Infant Transfer of Immunity - Previous studies demonstrated the transfer of immunity from a previously infected mother to offspring (ferrets). In the ferret, immunity was conferred by factors present in colostrum and milk. A preliminary experiment was performed in cebus monkeys to study maternal-to-infant transfer of immunity. Cebus monkeys were used because they are the only primate species available which could be sacrificed, and initial findings in cotton rats showed differential protection in the nose and lungs. Infants of seropositive and seronegative cebus monkeys were challenged with wild type RS virus, then sacrificed and examined for virus. The results, though preliminary, suggested that the lower respiratory tract of infants nursed by an immune mother was partially or totally protected, whereas the upper respiratory tract was not protected.

Additional data obtained in cotton rats confirmed last year's observations. That is, infants of immune cotton rats receive complete immunity to pulmonary infection, but incomplete immunity to nasal infection. This immunity is not long lived, and initial observations suggest that it may last only 4-5 weeks. Preliminary experiments with foster nursing indicate that the immunity is transferred through the milk, rather than the placenta. A related experiment involved the passive administration of RS virus antiserum, obtained from postinfection cotton rats, to infant cotton rats. Compared with control animals, which received an equivalent volume of normal cotton rat serum by the same route (intraperitoneally), animals which received antiserum showed significant resistance against subsequent pulmonary infection, but not against nasal infection.

Duration of Immunity Following Intranasal Infection of Cotton Rats - It is known that man does not develop lasting immunity to RS virus infection. During the past 18 months the transient nature of RS virus immunity was investigated in cotton rats. Immunity to nasal infection was temporary; animals were susceptible to nasal reinfection beginning 8 months after primary infection. The level of virus replication in the nose increased with time after initial infection; at 18 months post-infection animals

were completely permissive. In contrast, none of the animals examined throughout the course of the 18 month experiment were susceptible to reinfection of the lungs. If a similar situation obtains in man vaccination may offer greater promise of protection than had previously been considered possible.

Immunity in Cotton Rats Studied by Parabiosis - A surgical technique was devised by which animals were parabiosed and maintained alive for at least eleven days, the duration of the experiments. Since there are no inbred cotton rats, experiments were restricted to parabiotic pairs of same-sex littermates, in order to minimize immunological differences. Three types of pairs were formed: immune-to-immune (immune being animals parabiosed 21 days following intranasal infection by wild type RS virus), control-to-control, and immune-to-control. Immune-to-immune pairs were immune to reinfection both in the nose and the lungs. Control-to-control pairs, were susceptible to infection in both organs. Immune-to-control pairs, however, showed a surprising pattern. The immune partner remained immune to reinfection in both organs. The control partner, however, exhibited complete resistance in the lungs but not in the nose.

Intramuscular Immunization with Live RS Virus - Recently, Buynak and his colleagues at the Merck Institute for Therapeutic Research reported that parenteral administration of wild-type RS virus, grown in human diploid cells, induced the development of serum neutralizing antibody in young children without causing any objective signs or symptoms of disease. These exciting findings clearly required amplification and extension, because evidence that virus replicated following intramuscular (IM) inoculation was not provided in the original report, nor was the possible immunosuppressive effect of maternally derived passive immunity in parenteral immunization addressed. The former issue requires resolution because a virus preparation that failed to replicate and served only as an inactivated antigen might induce potentiation of disease. Since an RS virus vaccine is most urgently needed during the first few months of life, the issue of immunosuppression by maternally derived serum antibody represents a potential obstacle to success of the parenteral vaccine approach in this age group.

These two issues were examined using cotton rats as the experimental model of infection. This rodent is permissive for RS virus and supports a moderately high level of viral replication in the upper and lower portions of the respiratory tract.

First, the mechanism of induction of immunity was examined in an attempt to determine whether viral replication at the site of inoculation or within the respiratory tract was responsible for the resistance observed after IM inoculation of $10^{2.2}$ to 10^{4} PFU of live virus. Virus was not recovered from the local site of inoculation after 5 min. and was never detected in the nose or lungs at any time after IM inoculation. Furthermore, attempts to detect viral antigens at the site of IM inoculation were unsuccessful. However, inactivation of infectivity of three strains of

virus by the minimal UV dose required for inactivation markedly reduced or completely ablated their antigencitity and protective efficacy. Although this observation does not constitute unequivocal evidence for the occurrence of viral replication after IM inoculation, it suggests that limited replication, perhaps restricted to an abortive cycle, was responsible for stimulation of immunity by the small quantities of virus employed.

The issue of viral replication is of more than academic interest, because an inactivated, antigenic RS virus vaccine used previously in a series of trials did not stimulate immunity but did induce a state of altered reactivity such that disease was enhanced when vaccinees underwent natural infection. This disease potentiation effect could not be ascribed to tissue culture or medium constituents in the vaccine, since a comparison group of vaccinees who received a parainfluenza virus vaccine prepared in the same manner as the RS virus vaccine did not exhibit potentiation of disease when infected naturally with RS virus. Hence the mechanism by which a small quantity of live RS virus stimulated an immunological response must be resolved to be certain that it differs from that of inactivated vaccine. As cited above, this seems to be the case, since it appears that live virus undergoes limited replication following parenteral inoculation.

Second, the possibility that passive immunity might interfere with the effectiveness of parenteral immunization with live RS virus was examined because immunosuppression could pose a serious obstacle to this approach. Thus, the greatest need for an RS virus vaccine is in the first few months of life, a time when infants possess a moderately high level of maternally derived RS virus serum antibody. We attempted to simulate these conditions by administering live RS virus IM to weanling rats possessing passive serum antibody derived from their immune mothers. In this situation an immunosuppressive effect of passive immunity was observed. Only 50% of inoculated weanling rats were rendered resistant to subsequent IN challenge with RS virus. This suggests that parenteral immunization with live virus may not be effective in protecting human infants against RS virus during their period of greatest vulnerability to serious RS virus disease, i.e., the first 3 months of life. If this be the case, the usefulness of live IM virus vaccine may be limited to individuals over 6 months of age who have escaped natural infection and who have lost most or all of their passive maternally derived serum antibody. The outlook for IM vaccination of individuals who have undergone prior infection is not encouraging; Buynak's study indicated that seropositive children respond poorly to vaccine.

RS Virus Infection in the Immunosuppressed Host

Humans - Using immunoenzyme technology developed in this laboratory, which enables us to detect RS virus antigen in paraffin-embedded tissues, we have found that RS virus infection in the immunocompromised is far more

extensive than in normal individuals. In one immunodeficient infant and two immunosuppressed adults RS viral antigen was found in large amounts in the respiratory tract as well as other tissues. Dissemination of virus to non-respiratory tissues had not been recognized previously.

Cotton Rats - A regimen was devised whereby cotton rats received cyclophosphamide in small, repeated doses, namely 50 mg/kg three times a week. After three weeks, when the white cell count had dropped and stabilized at a reduced value, the animals were infected intranasally with RS virus. Drug treatment was continued on the same basis following infection. Viral titer in nose and lungs remained at maximal level as long as drug treatment continued (at least 58 days). In some animals virus was detected in the kidneys. In normal cotton rats, RS virus was never found beyond the respiratory tract.

Efficiency of coupling reactions and use of fluorescence substrates in enzyme-linked immunosorbant assays: Numerous enzyme-linked immunosorbant assays have been described using many different enzyme systems as well as many different methods of coupling or alternately, uncoupled enzyme conjugates. We have explored the effects of various coupling reactions using bi-functional reagents, periodate cross-linking procedures, and antibody enzyme soluble complex methods. In the evaluation of these coupling reactions three enzyme systems were employed: alkaline phosphatase, horseradish peroxidase, and glucose oxidase. The one-step glutaraldehyde coupling procedures appeared to be optimal for enzyme linked immunosorbant assay conjugates. Enzymatic sensitivities, when measured against concentrations of cholera toxin ranging from 10^{-7} to 10^{-12} grams/ml were approximately equal for the three enzyme systems when coupled with one-step glutaraldehyde procedure. This enabled the detection of 10^{-12} grams/ml of cholera toxin. The development of additional sensitivity in this assay system is under investigation with the use of fluorescence substrates.

Extension of ongoing enzyme-linked immunosorbant assay procedures: As reported last year we began to measured serum IgG antibody specific for respiratory syncytial virus. This year we have extended our capabilities to include the measurement of serum IgA as well as the IgA and IgG present in the nasal washings of patients. Current sensitivity levels in nasal washings are 4×10^{-12} grams/ml of IgA.

Immunohistoarcheology

This laboratory has been engaged in the development of a new method of enzyme-linked immunohistologic diagnostic technology. Because of the widely recognized difficulties in the evaluation of peroxidase staining due to endogenous peroxidase activity in tissues and inducibility of endogeneous peroxidase-like activity in inflammatory and neoplastic states we developed a method of enzyme-immunohistochemistry which would be unaffected by inflammatory and neoplastic processes. Our search for an enzymatic system not present in mammalian tissues led us to the consideration of

glucose oxidase, an enzyme derived from non-mammalian sources, as a possible candidate for this purpose. Earlier work had shown that by modification of the reaction product a stable preparation could be obtaind suitable for immunohistochemistry. Antisera were prepared against this enzyme in rabbits, guinea pigs and goats. These antisera were converted into a soluble enzyme antibody complex and used in a manner similar to that described by Sternberger. Currently we are determining the critical variables for respiratory syncytial virus antigen preservation during the fixation and embedment processes.

The initial application of this new methodology was in a cooperative study with the Center for Disease Control. After obtaining human material from which Legionella pneumophila was identified optimization of the testing procedure was conducted using visible bacteria as antigenic moities. After conclusion of this optimization procedure, 110 coded specimens of tissue, some from patients with legionnaires disease, were sent to us. We identified 19 specimens positive for Legionella pneumophilia. Upon breaking the blind code, there was complete correlation with the bacteriologic findings. Additional applications include antigenic analysis of the materials obtained from the 1978-1979 Naples, Italy epidemic outbreak as well as materials, obtained from many sources across the United States, of documented fatal RS virus illnesses. Additional testing, as well as production of infected tissue suitable for detection of respiratory syncytial virus, parainfluenza types 1, 2, 3 and 4, viruses and human rotavirus are currently underway. The methodology has already been extended to the detection of immunoglobulins G, A, and M, fibrinogen, albumin, and C3.

Experimental Aerosol Infection

Using the cotton rat as the susceptible host, infection via the aerosol route with respiratory syncytial virus has been undertaken. The virus is largely destroyed by the process, however, infections were established reproducibly in cotton rats and demonstrate that the cotton rat is sensitive to infection with as few as 25 plaque forming units per animal. Moreover, examination of the time course of infection by this means of inoculation with a small quantity of virus demonstrate that the peak titers occur not at day 4, as with intranasal infection, but at day 7 or later. Currently the effect of age and sex on viral titer are being examined.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00179-01 LID

PERIOD COVERED
OCTOBER 1, 1978 THROUGH SEPTEMBER 30, 1979

TITLE OF PROJECT (80 characters or less)
MOLECULAR BIOLOGY OF RESPIRATORY AND GASTROINTESTINAL VIRAL PATHOGENS STUDIED BY DNA RECOMBINANT TECHNOLOGY

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I. Ching-Juh Lai, Ph.D. Visiting Scientist LID, NIAID
Lewis J. Markoff, M.D. Medical Officer LID, NIAID

Associate Investigator: Brian R. Murphy, M.D., Medical Officer, LID,NIAID

COOPERATING UNITS (if any)
None

LAB/BRANCH
LABORATORY OF INFECTIOUS DISEASES

SECTION
RESPIRATORY VIRUSES SECTION

INSTITUTE AND LOCATION
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
48/12	24/12	24/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A procedure was devised for producing double-stranded DNA sequences corresponding to each of the influenza virus RNA segments. Negative and positive strands of influenza RNA segments were copied separately into DNA using the reverse transcriptase of avian myeloblastosis virus. The DNA products of the reverse transcriptase enzyme appeared to represent full-length genomic copies. The single-stranded DNA molecues from both preparations were annealed to generate double-stranded DNA segments which were subsequently isolated for cloning in plasmid PBR322 of E. coli K-12. The in vitro synthesized double stranded influenza DNA segments were than inserted into plasmic PBR322 at the specific Pst I site using the dG-dC tailing technique. The hybrid DNA molecues were used to transform recipient E. coli and transformants containing influenza gene sequences were identified by hybridization. In this manner, we have so far obtained putative full-length influenza virus DNA segments corresponding to genes coding for non-structural protein (Gene VIII), matric protein (gene VII), neuraminidase (gene VI), and hemagglutinin (gene IV). Cloning of other genes from wild type influenza A/Udorn/72 (H3N2) virus is now underway.

Objectives

(a) To employ recombinant DNA technology to study the genes of influenza virus and their functions; (b) to determine the antigenic determinants of the viral surface proteins and elucidate the pattern of antigenic variation among influenza virus strains by analysis of cloned DNA sequences; (c) to study the viral proteins synthesized in E. coli (harboring influenza virus genes) in relation to their antigenic properties; (d) to examine cloned viral DNA for gene expression in eukaryotic cells; (e) to develop procedures for conversion of cloned viral DNA back to the viral RNA with the intent of isolating or constructing mutants for use in experimental studies and possibly, immunoprophylaxis.

Methods

(a) Influenza viral DNA synthesis - Reverse-transcription of the viral RNA segments using (1) a specific DNA primer which is complementary to the 3'-end of virion RNA segments or (2) oligodeoxythymidylate primer.

(b) Cloning viral DNA copies in the E. coli-plasmid PBR322 system.

(c) Analysis of influenza DNA molecules by restriction enzyme cleavage and nucleotide sequencing.

(d) Radioimmunoassay for viral peptides synthesized in E. coli containing cloned influenza viral genes.

(e) Transfection or cloning of influenza viral DNA (from plasmid PBR 322) in eukaryotic cells using early region of SV40 as vector.

(f) Biochemical (nucleic acid hybridization), immunologic (radioimmunoassay or ELISA) and genetic (gene rescue of ts genes of defined ts mutants) analysis of transcription and expression of influenza genes in eukaryotic cells transformed by cloned influenza viral DNA.

Background

The genome of influenza virus consists of several separate RNA segments. The virion RNA segments are negative strands according to current nomenclature, that is, their complementary RNA strands become associated with the polyribosomes and are translated to produce viral specific proteins. So far, eight discrete virion RNA species have been identified by gel electrophoresis. Results from genetic complementation-recombination and biochemical analysis have demonstrated that each influenza RNA segments codes for a specific peptide product that is responsible for the respective viral function. Temperature-sensitive mutants in all eight complementation-recombination groups have been isolated and characterized.

Segmentation of the influenza viral genome is responsible for the high rate of recombination observed during coinfection by different influenza A virus strains within the same type. Recombination of influenza virus genes presumably occurs by free-exchange of RNA segments as a result of gene reassortment during infection. The mechanism of assortment that gives rise to a proper gene constellation in each infectious virus particle is not clear.

Another important feature of influenza virus is antigenic variation, or the emergence of viral subtypes (antigenic shift). Other phenomena involving antigenic variation of a lesser extent within a subtype (antigenic drift) are also observed. Such antigenic instability results in epidemiologically significant differences in surface antigens (hemagglutinin and neuraminidase) seen in the influenza A virus subtypes. When this type of change is abrupt and extensive (antigenic shift) a pandemic ensues. As a consequence, the continuing change of influenza strains has rendered ineffective efforts to vaccinate man against major antigenic variants.

In view of the medical importance of influenza viruses there is a need to broaden our knowledge of the genetics of these viruses in order to design ways to prevent pandemics. One approach is to use recombinant DNA technology that has been well-developed and has proven valuable in elucidating gene organization and expression in other appropriate host systems. Our general plan for this approach includes two phases. The first phase involves cloning each of the eight influenza gene segments using the E. coli K12-plasmid system (approved MUA #91). The second phase of development involves the use of purified clones of influenza DNA sequences to examine viral gene expression in animal cell culture and to produce vRNA in eukaryotic cells. To carry out this second phase we propose to utilize defective SV40 (lacking the late region of the genome that codes for capsid proteins) for construction of influenza-SV40 hybrid DNA molecules (submitted MUA #110). Introduction of the appropriate influenza-SV40 hybrid DNA molecules into eukaryotic cells may lead to transcription or translation of influenza viral DNA depending upon its orientation of insertion. Our goal is to devise procedures which would permit conversion of influenza DNA back to an influenza RNA negative strand and eventually transfer such RNA into an influenza virus. In this manner, stable, site-specific mutations, such as deletions, induced in the cloned DNA might be transferred back to the influenza virus. Thus, it may be possible to develop stable mutants for experimental study and for use in immunoprophylaxis.

Major Findings

(a) Influenza virus double-stranded DNA synthesis in vitro

In this study we devised a procedure for obtaining double-stranded DNA sequences corresponding to each of the influenza virus RNA segments. This approach should be generally applicable to negative-stranded RNA viruses containing segmented genomes. Specifically, the procedure involves separate

synthesis of DNA copies from both the negative and the positive strands of RNA segments using the reverse transcriptase of avian myeloblastosis virus. Influenza virion RNA segments, which are negative strands, were reverse-transcribed into their complementary DNA copies in the presence of a specific priming DNA oligomer. The primer is complementary to the conserved 3'-end sequence of the virion RNA segments and the DNA products of the reverse transcriptase enzyme appear to represent full-length genomic copies. Similarly, poly A-containing cytoplasmic RNA's (positive strands) isolated from the virus-infected cells were also transcribed to form DNA sequences in a reaction mixture in which oligo-(dT) primer was added. The single-stranded DNA molecules from both preparations were annealed to generate double-stranded DNA segments which were subsequently isolated for cloning in plasmid PBR322 of E. coli K-12.

(b) Influenza gene segments cloned in E. coli plasmid

As our first step in this project to obtain influenza DNA clones in a plasmid, the in vitro synthesized influenza DNA segments were inserted into plasmid PBR322 at the specific Pst I site using the dG-dC tailing technique. The hybrid DNA molecules were used to transform recipient E. coli and transformants containing influenza gene sequences were identified by hybridization. In this manner, we have so far obtained putative full-length influenza virus DNA segments corresponding to genes coding for the non-structural protein (gene VIII), the matrix protein (gene VII), the neuraminidase protein (gene VI), and hemagglutinin (gene IV). Cloning of other genes from wild type influenza A/Udorn/72 (H3N2) virus will be initiated shortly. Other experiments described in this report are in progress.

LABORATORY OF MICROBIAL IMMUNITY

1979 Annual Report
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PHS-NIH
SUMMARY STATEMENT

ANNUAL REPORT OF THE LABORATORY OF MICROBIAL IMMUNITY
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
October 1, 1978 to September 30, 1979

Richard Asofsky, M.D.
Chief, Laboratory of Microbial Immunity

Research in this Laboratory is concerned with the differentiation of lymphoid cells and with basic mechanisms controlling cellular and humoral immunity to microbial and tissue antigens. Selected microorganisms or their products are used in this work, and include viruses, blood parasites, bacterial polysaccharides and lipopolysaccharides. Tissue antigens include constituents of integrated leukemia viruses which are present on cell walls and histocompatibility antigens. Assembly and control of synthesis of immunoglobulins is studied in cloned lines of neoplastic plasma cells, B lymphocytes, or in somatic hybrids between neoplastic and normal cells. Somatic hybridization is also used to examine the repertoire of some antibody responses. Heterogeneity of thymus derived (T) cells is examined, using alloantigenic markers of T cell differentiation and certain lectins. Several genetic studies are in progress, including the identification and distribution in inbred mice of genes controlling leukemia viruses, those controlling differentiation of T lymphocytes, and those controlling antibody formation to pneumococcal polysaccharide.

I. IMMUNE RESPONSE GENES MAY BE ON DIFFERENT CHROMOSOMES

Previous studies conducted in this laboratory have shown that at least five autosomal genes influence the capacity of bone marrow-derived precursors of antibody-forming cells (B cells) to make an antibody response to Type III pneumococcal polysaccharide (SSS-III); these genes act in a complementary or additive manner and none appear to be linked to the major histocompatibility (H-2) or the immunoglobulin allotype complex. Congenic-resistant strains of mice having chromosomal segments from a high-responding strain on a low-responding background strain were used to isolate the genes involved and to assign such genes to well-defined genetic linkage groups or chromosomes. The results obtained showed that the introduction of segments from chromosomes 17, 9 and 4, as well as chromosome segments bearing the H-23 and H-27 loci appeared to increase immune responsiveness to SSS-III. Thus, genes governing the capacity of B cells to respond to SSS-III may be located on different chromosomes. It remains to be established whether the effects of these genes are specific for the antibody response to SSS-III or influence responsiveness to other antigens.
(Dr. P. J. Baker; B. Prescott; Mr. G. Caldes; Ms. D. F. Amsbaugh; and P. W. Stashak)

II. THYMUS-DERIVED HELPER CELLS REQUIRED FOR INCREASED IMMUNOGENICITY OF THE HIPOTEICHOIC ACID OF STAPHYLOCOCCUS AUREUS

The antibody response to the lipoteichoic acid (LTA) of S. aureus was examined by a newly developed procedure in which erythrocytes, sensitized with periodate-activated LTA, were used for the detection of serum antibody and antibody-producing plaque-forming cells (PFC). Serum antibody and PFC were found in mice immunized with heat-killed S. aureus. The specificity of such antibody was affirmed by inhibition tests using purified LTA; here, larger amounts of unrelated polysaccharide and protein, extracted from S. aureus membranes, were without effect. LTA-specific PFC were first detected two days after immunization with heat-killed bacterial cells; maximal numbers were attained by day four. But, no PFC were found in mice given purified LTA over a 10,000-fold range of immunizing doses. Prior immunization with LTA did not reduce the PFC response to heat-killed bacterial cells; thus LTA was not tolerance-inducing. Mice pretreated with a carrier known to activate thymus-derived (T) helper lymphocytes produced a PFC response to LTA only when immunized with LTA bound to the same carrier. This suggests that carrier-specific T cells are needed to initiate an antibody response to LTA. Since an antibody response can be elicited in mice given killed bacterial cells, other cell wall and/or cell membrane constituents may play an important role as immunologically active carriers in this regard.
(Dr. P. J. Baker, Dr. B. Prescott; Mr. G. Caldes, Ms. D. F. Amsbaugh; and Mr. P. W. Stashak, with Dr. P. R. Beining and Ms. G. M. Flannery (Univ. Scranton).

III. INTRATHYMIC DIFFERENTIATION OF T CELLS STUDIED

Examination of thymocyte subpopulations fractionated by peanut lectin agglutination has identified functionally and phenotypically distinct subsets of cells. These subsets are presumably precursors for peripheral T cells with similar function and phenotype. Initially, a minor subset of phenotypically differentiated Lyt 1^+23^- cells were demonstrated in the thymus with immunofluorescence by flow microfluorometry (FMF). Using peanut lectin fractionation, it was shown subsequently that this population could be enriched from 10% in a population of normal thymocytes to 50-70% of peanut non-agglutinated (PNA^-) thymocytes. PNA^- thymocytes consist of a population roughly comparable to cortisone resistant thymocytes in size and mitogen reactivity. In addition to the enrichment for Lyt 1^+23^-, in the PNA^- cells, a population of Lyt 123^+ cells is consistently observed in the PNA^- fraction. Subsequent analysis of this Lyt 123^+ fraction has demonstrated a further subset of these cells which are eliminated with an antisera to a marker, CTL, on cytotoxic lymphocytes and cytotoxic lymphocyte precursors. The PNA^- cells which are eliminated with this antisera account for part but not all of the cytotoxic precursor activity, while all peripheral differentiated T lymphocytes with cytotoxic precursor activity can be eliminated with this antisera. These fundings suggest the presence of a differentiation pathway in which both phenotype and function for cytotoxic lymphocytes are developed in a subset of PNA^- Lyt 123^+ thymocytes. These analyses have demonstrated further

that extremely small populations, in this case less than 1% of the normal thymocytes, can be identified with suitable pre-enrichment techniques and FMF analysis. (Drs. B. J. Mathieson, R. Asofsky, I. Betel, and M. Mage (NCI), Ms. P. Campbell and S. O. Sharrow (NCI)).

IV. GENETIC STUDIES OF MOUSE LEUKEMIA VIRUSES

Murine leukemia viruses (MuLV) are highly polymorphic. The mouse genome includes about 50 sequences related to the genetic information coding for the different classes of MuLV (termed ecotropic, xenotropic and amphotropic). Some of these sequences code for complete infectious MuLV of the various classes whereas some code for only isolated portions of the intact virus. Studies of xenotropic MuLV (X-MuLV) present in all strains of mice, have shown that expression of X-MuLV as infectious virus or as virus coded gp70 on the surface of lymphocytes varies considerably from strain to strain (Morse, Chused, Boehm-Truitt, Mathieson, Sharrow and Hartley) and in different lymphoid tissues (Morse, Chused, Sharrow and Hartley) and that expression in these two modes is not coordinate.

Tests for infectious virus demonstrated that NZB mice have two loci coding for X-MuLV and that, as for ecotropic MuLV, these loci are dominant in crosses with NFS and other strains of mice (Chused and Morse). By comparison, F/St mice appear to have a single locus for infectious X-MuLV with the locus behaving as a recessive in genetic crosses with several strains of mice. Furthermore, NZB's produce 10-fold more X-MuLV in spleen than thymus whereas the ratio is reversed in F/St spleen and thymus (Morse, Chused, Sharrow, Hartley; Morse, Wolford, Hartley-unpublished). Finally, in NZB's, the levels of infectious X-MuLV produced by T cells is affected by genes governing T cell differentiation - thymocytes produce less virus than peripheral T cells. (Morse, Chused, Sharrow and Hartley).

Analyses for X-MuLV cell surface antigen expression (gp70's termed XenCSA) showed that disparate levels of XenCSA in strains DBA/2 (high XenCSA) and C57BL/6 (B6) are governed by a semidominant gene on chromosome 4 at or near Fv-1 (Morse, Chused, Taylor, Hartley and Sharrow). These results have been confirmed in crosses between B6 and the high XenCSA strain C3H and have been extended to show that XenCSA is under independent genetic control from another cell surface gp70 marker, GIX. Increasing awareness of the biology of X-MuLV should be of great help in defining their role in leukemogenesis, particularly in regard to the formation of recombinants with ecotropic MuLV. (Morse, Stockert, Chused, Obata, Kozak and Taylor).

V. IgD ON B-CELL LYMPHOMAS

Five lymphomas were identified previously, which had cell surface immunoglobulin, as indicated by staining with fluorescein-labeled anti-immunoglobulin. Cell surface IgM was found on four; L10A, X16C, K46, and BAL 17. These cell lines also synthesize but do not secrete monomeric IgM. Fluorescein-labeled anti-δ stains all five lines. The presence of IgD has also been shown by labeling surface molecules with ^{125}I, then precipitating cell lysates with anti-δ, and analyzing the labeled products by polyacrylamide gel electrophoresis in SDS. Finally these cell lines synthesize, but do not secrete IgD. (Drs. Kim, McKeever, Laskov (not NIH) and Asofsky; Mr. Nero).

VI. NONSPECIFIC SYNTHESIS OF IMMUNOGLOBULIN STUDIED

When animals are immunized with many antigens, increased synthesis of immunoglobulin (Ig) far exceeds the synthesis of specific antibody. The reverse-plaque assay has been used to examine this increase. Isotype-restricted antisera were used to develop the reverse plaques. Certain mitogens such as E Coli LPS cause increases largely in IgM producing cells. Primary infection with nonlethal Plasmodium yoelii produces a parallel increase in all classes, whereas secondary infection produces increases mainly in the IgG classes. Stimulation with aggregated human IgG or sheep red blood cells cause increase mainly in IgGl. Animals deprived of T cells did not show these increases (except for stimulation with LPS). The use of more than one antigen has shown that the precursors of the reverse plaques must belong to sets of B lymphocytes which overlap but which are not identical. It therefore appears that nonspecific stimulation of B cells occurs via stimulation of T cells (Drs. Rosenberg and Chiller (not NIH); Drs. Rosenberg, Taylor, Weinbaum and Mr. Evans).

ADMINISTRATIVE

Dr. Thomas M. Chused joined the Laboratory this year. He has continued his research on autoimmune diseases. The NIAID fluorescence-activated cell sorter has been installed together with a PDP-11/34 computer. The sorter is a two-laser, 2 color instrument. Dr. Chused is now programming the computer to analyze, display and print data from experiments. Also joining the Laboratory this year were Dr. Diane W. Taylor, a Staff Fellow, Dr. Wendy F. Davidson, a Visiting Fellow and Drs. Janice Longstreth, Florence Rollwagen and Joyce Schroer, all NIH post-doctoral fellows. Dr. Sanford Stone was moved in July from OSD to LMI where he will be head of the Section on Experimental Autoimmunity.

Dr. Paul E. McKeever completed three very productive years as a Research Associate. He is now a senior investigator in the Neurosurgery Branch, NINCDS. Dr. Carol Ludwig completed her associateship, and is now with OD, NIA.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00134-17 LMI

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Control of Immunoglobulin Synthesis in Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	K. J. Kim	Visiting Associate	LMI/NIAID
	C. Kanellopoulos-Langevin	Visiting Associate	LMI/NIAID
	Y. J. Rosenberg	Visiting Fellow	LMI/NIAID
	C. B. Evans	Biological Lab Tech	LMI/NIAID
	B. J. Fowlkes	Microbiologist	LMI/NIAID
	W. J. Logan	Biological Lab Tech	LMI/NIAID
	G. B. Nero	Biological Lab Tech	LMI/NIAID
	G. M. Shearer	Senior Investigator	IB/NCI
	R. B. Levy	Post-doctoral Fellow	IB/NCI
	D. H. Sachs	Section Head	IB/NCI
	R. Laskov*		
	J. M. CHiller**		

COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Microbial Immunity

SECTION

Experimental Pathology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
72/12	54/12	18/12

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

SAMM 368, a myeloma which secretes IgG2b and IgA paraproteins simultaneously, shows cytoplasmic segregation of the γ and α heavy chains, and rapid assembly of IgG2b monomers and IgA multimers, the latter bonded to J chains in a late stage of assembly.

Several B-cell tumors have surface-IgD as well as surface IgM by immunofluorescence. Synthesis of IgD has been demonstrated in these tumors.

PHS-6040
(Rev. 10-76)

Project Description:

As in the past several years, considerable attention was given to studies of neoplastic lymphoid cells. Work was also done on the control of synthesis of immunoglobulins of different classes (isotypes) following stimulation of mice with different antigens.

A. Intracellular Assembly of Two Classes of Immunoglobulin in Individual Plasma Cells

Individual plasma cells of the myeloma SAMM 368 spontaneously secrete IgA and IgG2b in the absence of experimental hybridization. Immunoglobulin biosynthesis and secretion by cell lines established in vitro from SAMM 368 was studied. The cells secrete IgA predominantly as multimers with disulfide bonded J-chains and noncovalently bonded light chains. They simultaneously secrete IgG2b only as a monomer having two disulfide bonded light chains. The similarities in subunit composition made by this plasmacytoma and single immunoglobulins of the same isotypes show that a cell can fully assemble and secrete two multichain immunoglobulins in a manner similar to cells which secrete single immunoglobulins. SAMM 368 synthesizes and secretes fully assembled IgG2b more rapidly than IgA, and secretes larger amounts of IgG2b than IgA, suggesting regulatory factors of Ig synthesis in addition to those used by cells secreting single immunoglobulins.

The γ2b and α-chains of SAMM 368 are segregated to the appropriate immunoglobulin isotype, thereby avoiding secretion of γ2b-α mixed molecules. Similar segregation of heavy chains occurs among cytoplasmic precursors of IgG2b and IgA. This indicates that segregation of heavy chains occurs at an early stage in biosynthesis prior to the point of regulation by proteolytic enzymes. J-chains is added late in biosynthesis only to the IgA. The reliability and speed with which SAMM 368 synthesizes two complete multichain proteins without making inappropriately mixed molecules favors chemical conformation and cellular compartmentalization as factors critical to biosynthesis of two immunoglobulins by a single cell.

Two different κ-chains are present on the IgA and IgG2b of SAMM 368 growing in vivo. IgA and IgG2b secreted in vitro has similar κ-chains with major isoelectric point of 6.8, possibly reflecting different regulatory factors in vitro than in vivo. (Drs. McKeever and Asofsky, Mr. Nero (student))

B. B Cell Tumors

Previously, we reported the establishment of six BALB/c B cell lymphoma lines expressing different surface markers: (1) K46, L10A, X16c and BALENTL 17 are IgM^+ Ia^+ Fc^+, C3 $receptor^-$, (2) A20 is Ig^+ Ia^+, Fc^+, C3 $receptor^-$. The presence of S-IgD from these tumors was indicated by analysis of surface immunoglobulins following labeling of surface molecules with ^{125}I using the lactoperoxidase method. Further, flow microfluorometry following staining with

several kinds of fluorescein-labeled anti-δ antibodies, including a hybridoma product, showed IgD on the surface of cells of at least four cell lines. Tumors K46, X16C and L10A were induced to secrete IgM when fused with a drug-marked IgG2b producing MPC-11 myeloma cell line. There was no suppression of the parent IgG2b synthesis in the hybrids. (Drs. Laskov, Kim and Asofsky).

Biosynthesis and surface expression of IgM and IgD has been studied in spontaneous lymphomas of BALB/c mice. Lymphomas K46 and L10A synthesize IgM monomers and halfmers. A molecule with the molecular weight expected of IgD is usually detected by biosynthetic labelling. This molecule was best detected with anti-δ antisera and sensitive radioiodine surface labelling techniques. Light chains on the IgM and IgD of K46 have similar isoelectric points. The light chains attached to IgM halfmers have different isoelectric points than light chains on IgM monomer of K46, suggesting post-translational modification of this light chain. (Drs. McKeever, Kim and Asofsky, Mr. Nero (student))

We have also pursued the work on B cell lymphoma lines as models for the study of normal B cell subpopulations since these in vitro cell lines express different surface immunoglobulin classes and several other B cell markers including Ia and LyM antigens, and Fc (IgG) receptors. Three different cell lines were selected for further investigation of the possible relationship between Fc (IgG) receptors and alloantigens on their surface. Three different patterns of inhibition by the alloantisera tested (anti-Ia, anti-H-2D and anti-LyM) were observed. Further studies of the mechanism(s) of the blocking of Fc (IgG) receptors by all antibodies have been delayed by the difficulty in obtaining reasonable amounts of $F(ab')_2$ or Fab fragments of mouse alloantibodies after digestion. Inhibition experiments performed in the presence of sodium azide or after mild reduction and alkylation of alloantibodies suggest that these inhibitions are not due only to Fc portions of alloantibodies. Mixing experiments using A20 (Ia^+) and M12 (Ia^-) mixed 1/1 and incubated with anti-Ia serum resulted in a 50% inhibition, suggesting that alloantibodies block Fc receptors only on the cell to which they are bound. The differences we have observed between the relationships of alloantigens and Fc receptors in these cell lines may reflect their origin from different distinct B cell subpopulations.

C. T Cell Tumors

Ten BALB/c T cell lines which expressed restricted patterns of Lyt differentiation antigens were investigated for the possible immunological functions: Two Lyt 1^- Lyt 2^+ cell lines, BALENTL 4 and 14, gave significant suppression of the MLR between BALB/c and C57BL/6. Furthermore, BALENTL 14 also inhibited the generation of BALB/c effector cells against C57BL/6 spleen cells. (Drs. Kim, Weinbaum and Asofsky)

Three T cell lines, BALENTL 4 (Ly $1^{-}2^{+}$), BALENTL 9 (Ly $1^{+}2^{+}$) and BALENTL 13 (Ly $1^{+}2^{-}$), were examined for their sensitivity to glucocorticoids and their receptor content. All these lines were found to be sensitive to dexamethasone and to contain (high glucocorticoid receptors). (Drs. Schmidt, Kim and Thomas)

D. Hybridomas

Spleen cells from BALB/c mice immunized with SSS-III were fused with drug marked myeloma cell lines (NS-1, or 45.6TG 1.7 cell line). Several stable hybrids secreting monoclonal antibodies specific for SSS-III were obtained. (Drs. Kim and K. Schroer)

See Project No. Z01 AI 00149-09 LI - Hybridomas Producing Antibody to Insulin.

E. Other Studies With Tumors

The possiblity of lymphotoxin release from the established T-, B-, macrophage cell lines, were determined by measuring the ^{3}H-thymidine release from a fibroblast cell line, P7943. Macrophage lines (J774.1, P388D1, PU5-1.8 and J774.1) secreted the cytotoxic material, however, none of our B and T lymphoid lines gave any positive results.

F. Control of "Nonspecific" Synthesis of Different Isotypes of Immunoglobulin (Ig) Following Immunization

Using a reverse plaque-forming cell (PFC) assay which detects total Ig secreting cells or those secreting Ig of one particular class, regardless of specificity, in vivo experiments have been done to study mechanisms which result in activation of B cells. It is shown that the isotype of the induced PFC clearly reflects the cellular requirements for stimulation of B cells in the different cases. For example, the mitogen LPS which requires only minimal signals from accesory cells, results in early increases in PFC restricted to the IgM class. In addition, polyclonal B cell activation can occur via two different T-cell dependent mechanisms. First, during malaria infections, a marked B cell activation occurs. Analysis of this response reveals that all PFC classes increase in a parallel fashion suggesting a role of T cell derived lymphokines which act non-specifically on all B cell precursors. Reinfection of mice previously given malaria however results in preferential activation of IgG classes. The second form of non-specific B cell activation occurs following either immunization with classical T-dependent antigens, e.g. sheep erythrocytes, agg. human gamma globulin, or heterologous serum treatment and requires the participation of T helper cells specific for inducing antigen. In this case, the non-specific increases are class restricted, responses occuring predominantly in the IgG (particularly IgG1) class and, in one case, also IgA.

It is of particular interest that the Ig secreting cell populations induced by each antigen belong to overlapping sets of cells. The finding of class-specific increases in non-specific PFC is not limited, and has been shown using hapten-carrier conjugates in vivo and in vitro, as well as the above listed antigens. These studies are now aimed at the demonstration of isotype-specific helper cells, and the possible recognition of isotypes and idiotypes. Selective recognition may cause restricted stimulation of B cells. (Drs. Rosenberg and Chiller (not NIH))

G. Immunoglobulin Production in CBA/N Mice.

CBA/N mice bear a sex-linked (X-linked) genetic defect which precludes antibody responses to several polysaccharide antigens and results in decreased numbers of IgM secreting cells with a parallel decrease in levels of serum IgM. This genetic defect is most dramatic in (CBA/N X NZB)F_1 male mice, which express the defect fully, despite unusually high IgM levels seen in the paternal NZB strain. Recent work has shown that this defect can also regulate respones to autologous erythrocyte antigens as assayed on bromalein-treated isologous RBC, but that this response can be largely regained in older mice. The results suggest that the defective mice suffer from low frequencies of B cell precursors specific for certain antigens rather than the complete absence of B-cell population. (Dr. Rosenberg)

H. Immunoglobulin and Antibody Synthesis Following Intratracheal Stimulation with Antigen

Sheep red blood cells or SSS-III polysaccharide were administered to BALB/c mice by intratracheal intubation. Systemic antibody responses were almost absent in animals immunized by this means, as demonstrated by absent or very low specific plaque-forming cells (PFC) in spleens. Draining (bronchial) lymph nodes showed substantial PFC responses between days four and twelve of immunization similar in magnitude to PFC responses in spleen after systemic immunization. Antibody belonging to all classes (isotypes) was seen, without significant preference for IgA as opposed to IgG. (Drs. Munoz and Asofsky)

Publications:

Kim, K. J., Weinbaum, F. I., and Asofsky, R.: Functional characteristics of BALB/c T cell lines: Suppression in the mixed lymphocytes response and the cell mediated lysis. J. Immunol. 121: 2299-2304, 1978.

Kim, K. J., Kanellopoulos-Langevin, C., Merwin, R., Sachs, D., and Asofsky, R.: Establishment and characterization of BALB/c lymphoma lines with B cell properties. J. Immunol. 122: 549-554, 1978.

Laskov, R., Kim,K. J., and Asofsky, R.: Induction of amplified synthesis and secretion of IgM by fusion of murine B-lymphoma with myeloma cells. Proc Natl. Acad. Sci. 76: 915-919, 1978.

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Aksamit, R. R., and Kim, K. J.: Macrophage cell lines produce a cytotoxin. J. Immunol. 122: 1785-1788, 1979.

Levy, R. B., Shearer, G. M., Kim, K. J., and Asofsky, R.: Xenogenic serum-induced murine cytotoxic cells: I. Importance of the serum source in the generation of primary cytotoxic effectors without the addition of stimulation cells. Cellular Immunol. (In press) 1979.

Schmidt, T. M., Kim, K. J. and Thompson, E. G.: Glucocorticoid sensitivity and receptors in BALB/c T cell lymphoma lines expressing restricted patterns of Ly differentiation antigens. J. Steroid Biochemistry. (In press) 1979.

McKeever, P. E., Kim, K. J., Nero, G. B., Laskov, R., Merwin, R. M., Logan, W. J., and Asofsky, R.: Two spontaneous BALB/c lymphomas synthesize IgM; μ_2L_2 and µL molecules are isolated and characterized while another molecule resembles IgD. J. Immunol. 122: 1261-1265, 1979.

McKeever, P. E., Neiders, M.E., Nero, G. B., and Asofsky, R.: Murine plasma cell tumors secreting more than one class of immunoglobulin. VI. Secretion of completely assembled IgG2b and IgA molecules with segregated heavy chains and free light chains by spontaneous myeloma SAMM 368 in culture. J. Immunol. 122: 1972-1977, 1979.

Rosenberg, Y. J. and Chiller, J. M.: The ability of antigen-specific helper cells to effect a class-specific increase in total Ig secreting cells in spleens after immunization with the antigens. J. Exp. Med.. (In press) 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00135-05 LMI

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Properties of Immunoglobulin Secreting Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. C. Morse III	Senior Investigator	LMI/NIAID
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	M. Potter	Head, Immunochemistry Sect.	LCBGY/NCI
	R. Riblet*		
	M. Weigert		
	O. Makela**		
	R. Lieberman	Senior Investigator	LI/NIAID
	B. Birshstein***		
	T. Mossman****		

COOPERATING UNITS (if any) *Institute for Cancer Research, Fox Chase, Philadelphia, PA
**University of Helsinki, Helsinki, Finland
***Albert Einstein College of Medicine, Bronx, N.Y.
****University of Alberta, Alberta, Canada

LAB/BRANCH
Laboratory of Microbial Immunity

SECTION
Experimental Pathology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7/12	3/12	4/12

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A large series of NZB plasmacytomas was studied to determine the classes of immunoglobulin produced. The time course for plasmacytoma development and the frequency of immunoglobulin classes produced differed significantly from tumors produced in BALB/c mice. Unique immunoglobulins carrying markers of both IgG3 and IgA immunoglobulin classes were detected.

SAMM 368 is a BALB/c plasmacytoma secreting two classes of immunoglobulin - IgG2b and IgA - which do not share idiotypes. The IgG2b molecule does not express the appropriate allotype markers for the class. Studies of this protein suggest that allotype markers for IgG2b are expressed by the CH_3 domain.

PHS-6040
(Rev. 10-76)

Project Description:

NZB mice were induced to form plasmacytomas by the intraperitoneal injection of pristane. Mice were followed for the time course of plasmacytoma development, the immunoglobulin classes produced by each tumor, and the capacity of these immunoglobulins to react with selected haptens.

NZB plasmacytomas, as compared to BALB/c tumors were 1) found to require longer to develop after pristane injection; 2) express a much higher frequency of IgG's (IgG1, IgG2a, IgG2b, IgG3) and a lower frequency of IgA's; and 3) produced unique molecules never found in BALB/c, which have characteristics of both IgA and IgG3 immunoglobulins. Peptide maps of the heavy chains from these IgA-IgG3 "doubles" suggest that there may be peptides unique to this set of paraproteins which are otherwise very much like IgA's.

The NZB paraproteins as well as some from BALB/c mice were tested for their capacity to bind a series of haptens. A large number of new hapten-binding immunoglobulins were detected.

A BALB/c plasmacytoma, SAMM 368, produces an IgG2b molecule which lacks appropriate allotypic markers for its class of immunoglobulin. Studies of other myeloma variants lacking the CH_3 domain of IgG2b or with a substitution of the CH_3 of IgG2b with that of IgG2a indicate that the CH_3 domain carries the IgG2b allotype markers. This portion of the SAMM 368 heavy chain is being sequenced along with a normal IgG2b heavy chain to determine the structural basis for allotype expression.

Publications:

Morse III, H. C., Riblet, R., Asofsky, R., and Weigert, M: Plasmacytomas of the NZB mouse. J. Immunol., 121: 1969-1972, 1978.

Makela, O., Kaartinen, M., Karjalainen, K., Morse III, H. C., Weigert, M., and Potter, M.: A search for hapten-binding mouse plasmacytoma proteins. Eur. J. Immunol., 9: 125-129, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AI-00136-07 LMI

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Functional Activities of Subpopulations of Thymus-Derived Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	S.O. Sharrow	Chemist	IB/NCI
	I. Betel*		
	M. Mage	Senior Investigator	LB/NCI
	K. Bottomly**		
	B. J. Fowlkes	Microbiologist	LMI/NIAID
	C. Kanellopoulos-Langevin	Visiting Fellow	LMI/NIAID
	M. Potter	Head, Immunochemistry Sect.	LCBGY/NCI
	M. Timmons	Co-op Student	LMI/NIAID
	L. Morrow	Student	LMI/NIAID
	F-W Shen***		
	E. A. Boyse***		

COOPERATING UNITS (if any)

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** The Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia,Pa.
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LAB/BRANCH

Laboratory of Microbial Immunity

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
23/12	13/12	10/12

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To examine subpopulations of thymocytes, we have extended the use of flow microfluorometry (FMF) analysis to characterize cell surface and functional parameters of peanut lectin agglutinated cells. In addition to analysis for known antigenic markers, e.g. the Lyt antigens, we have extended the analysis of these cells using fluorescent lectin probes. We have currently undertaken analyses of the functional differentiation of these thymocyte subpopulations for cell-mediated cytotoxicity and antigen, mitogen, or monokine induced proliferation, to assess the extent of functional differentiation determined by the thymus.

A contaminant antibody detected by FMF in the antisera from several non-congenic Lyt immunizations has been examined and used to characterize and define a new lymphocyte cell surface antigen Ly 9 which distinguishes lymphocyte populations from all other tissues and cells. Since this marker is expressed on lymphocytic precursors, as well as differentiated lymphocytes, and not on erythrocytic precursors or differentiated red blood cells, it may prove useful for the study of functional lineages of bone marrow precursors.

Project Description:

1. (A) Expression of T-cell Differentiation Antigens on Normal Thymocytes.

Flow microfluorometry analysis of thymocyte subpopulations has been extended for Lyt antigens and other cell surface differentiation markers. Using a pre-enrichment of thymocyte subpopulations with peanut lectin agglutination, we have analyzed the possibility that cell surface differentiation seen in a small subset of cells in the thymus reflects functional competence of the cell types in that organ.

Peanut lectin agglutination allows the positive enrichment of 2 subsets of thymocytes with different Lyt phenotypes. Peanut lectin agglutinated (PNA^+) cells are nearly all Lyt 123^+, high Thy-1, while non-agglutinated (PNA^-) cells are highly enriched (to about 60%) for low Thy-1, Lyt 1^+23^- thymocytes. Cells with this latter phenotype constitute only about 10% of normal thymocytes. PNA^+ and PNA^- thymocytes have been analyzed co-ordinately for Lyt phenotype and cytotoxic T cell function. More recently an additional cell surface differentiation marker, CTL, expressed on cytotoxic lymphocytes and their precursors has been used in these analyses.

(1) The analysis of cytotoxic T lymphocyte precursors (CTLp) has demonstrated that following: (a) Although Lyt 1^+23^- cells are enriched in the PNA^- fraction, the Lyt 123^+ cells in that fraction are both necessary and sufficient for generation of cytotoxic function. (b) There is a subset of Lyt 123^+ cells which are differentiated for the CTL marker. This subset corresponds to about 6% of the PNA^- cells or about 0.6% of the starting normal thymocyte population. Detection of this population is only possible with the pre-enrichment procedure and indicates the presence of a differentiation sequence, wherein an Lyt 123^+ thymocyte acquires the CTL marker and functional competence.

(2) In collaboration with Dr. J. Oppenheim's laboratory, NIDR, using human monocyte-derived lymphocyte activation factors, (LAF), we have analyzed the differential effect of isolated factors on the Lyt phenotype and on induced proliferative activity of thymocytes, cortisone resistant thymocytes and PNA^- and PNA^+ thymocyte subpopulations. These analyses have demonstrated that the conventional lymphocyte activating factor (LAF) of 14,000 daltons, which is highly active for stimulation of thymocyte proliferation, does not cause a phenotypic shift in Lyt antigens. However, a higher molecular weight factor (>60,000 daltons), isolated from human monocyte, LPS stimulated cultures, not only induces proliferation in thymocytes, but also causes a quantitative increase in the expression of Lyt 1 antigen on the cell surface of thymocytes exposed to the factor in vitro. This increase in Lyt 1 expression is seen at 12-18 hr, prior to the blastogenic increase in cell size. This phenotypic shift also precedes the mitogenic activation by the factor which is measured by tritiated thymidine uptake at 48 hr. Both PNA^- and PNA^+ cells display this increase of Lyt 1 antigen in response to the high molecular weight LAF, thus indicating no correlative parallel selective activity on fractionated thymocyte subpopulations.

(3) As an alternative to antibody probes to cell antigens, we are currently examining by FMF a number of plant and animal lectins capable of recognizing complex sugar moieties on glycoprotein or glycolipid structures of the cell surface. Following our successful application of peanut lectin fractionation to enrich minor subpopulations, we intend to expand to the use of several other lectins. We are attempting to further identify and subdivide thymocyte subpopulations, for which there is evidence by physical fractionation, cell surface antigenic characterization or functional heterogeneity. These several aspects are being investigated in collaboration with Dr. M. J. Waxdal (See project Z01-AI-00038-07 LI).

II. (A) Genetic Analysis of Lymphocyte Differentiation Markers

A new lymphocyte cell surface alloantigen, provisionally designated Ly 9, is detected as an extra specificity by flow microfluorometry (FMF) in sera from anti-Lyt immunizations. Ly 9.2, one of the apparently allelic specificities commonly is detected in anti-Lyt 3.1 immunizations; (C3H/HeN X SJL/J)F_1 anti-C58 normal thymocytes. The alternative antigen expression, Ly 9.1, can be detected routinely in sera from two other Lyt immunizations; C57BL/6-H-2^k anti-CE/J normal thymocytes (anti-Lyt 2.1), and C58 anti-CE normal thymocytes (anti-Lyt 3.2). This thymocyte alloantigen has both a unique strain distribution and a unique cell/tissue distribution, differing from previously reported cell surface antigens. Ly 9 is expressed on all thymocytes, lymphocytes and apparently on lymphocyte and/or white cell precursors in the bone marrow. This antigen is not expressed to any significant degree on erythrocytes, epidermal cells, sperm, testis, brain, kidney, liver or lung. FMF analysis and absorption typing reveals a quantitative difference between the level of antigen expression on cells from thymus versus spleen or lymph node. Cytotoxic elimination experiments confirm that Ly 9 is expressed on both T and B cells and on at least two different T cell functional subsets.

(B) We have continued to analyze the genetic inter-relationships of the (NZBXC58) recombinant inbred lines for lymphocyte cell surface antigen markers.

Publications:

Mathieson, B. J., Sharrow, S. O., Campbell, P. S., and Asofsky, R.: An Lyt differentiated subpopulation of thymocytes detected by flow microfluorometry. Nature 277: 478-480, 1979.

Betel, I., Mathieson, B. J., and Sharrow, S. O.: Differential agglutination of thymocytes by peanut agglutinin: Phenotypic charcterization of the subpopulations by flow microfluorometry. In Petters, H., (Ed): Protides of the Biological Fluids. Pergammon Press, Oxford. In Press, 1979.

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Bottomly, K., Mathieson, B. J., and Mosier, D. E.: Anti-idiotype induced regulation of helper cell function for the response to phosphroylcholine in adult BALB/c mice. J. Exp. Med.. 148: 1216-1227, 1978.

Bottomly, K., Mathieson, B. J. Cosenza, H., and Mosier, D. E.: Idiotype specific regulation of the response to phosphorylcholine by T cells from mice with high and low levels of circulating idiotype. In Cooper, M., Mosier, D., Sher, I., and Vitteta, E., (Eds.), B Lymphocytes in the Immune Response. Elsevier North-Holland, New York. (In press) 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00137-13 LMI

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Biology of Graft-Versus-Host Reactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: R. Asofsky Laboratory Chief LMI/NIAID

COOPERATING UNITS (if any)

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Laboratory of Microbial Immunity

SECTION
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NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0/12		

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

No progress was made this year. Studies of the "allogeneic effect" - see Z01 AI 00145-12.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00138-05 LMI

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Viruses and the Immune Response

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Herbert C. Morse III	Senior Investigator	LMI/NIAID
Other:	T. M. Chused	Senior Investigator	LMI/NIAID
	J. W. Hartley	Senior Investigator	LVD/NIAID
	S. O. Sharrow	Biologist	IB/NCI
	W. F. Davidson	Visiting Fellow	LMI/NIAID
	J. H. Goode	Biologist	LMI/NIAID
	N. K. Wolford	Biologist	LVD/NIAID
	B. A. Taylor*		
	J. D. Longstreth	NIH Postdoctoral Fellow	LMI/NIAID

COOPERATING UNITS (if any)

*The Jackson Laboratory, Bar Harbor, Maine

LAB/BRANCH

Laboratory of Microbial Immunity

SECTION

Experimental Pathology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
36/12	28/12	8/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Genetic analyses of xenotropic murine leukemia viruses in inbred mouse strains have shown that these viruses may be expressed as infectious virus or as virus-coded antigens (gp7-'s termed XenCSA) on the surface of normal lymphocytes. NZB mice have two independently segregating dominant loci for infectious virus expression and apparently one coding for XenCSA in the absence of infectious virus. The levels of virus expression as infectious virus and XenCSA in NZB lymphocytes is governed by genes affecting lymphocyte differentiation. In genetic crosses of mice exhibiting high (DBA/2) and low (C57BL/6) levels of XenCSA, a single semidominant gene on chromosome 4 at or near Fv-1 has the predominant effect on XenCSA levels.

PHS-6040
(Rev. 10-76)

Project Description:

Several strains of mice are being studied to determine genetic, developmental and environmental factors affecting the expression of xenotropic murine leukemia viruses (X-MuLV) as infectious virus or as virus-coded antigen (gp70) in the presence or absence of infectious virus. Strains producing high levels of infectious virus include NZB, F, and BXD-14, a recombinant inbred derived from low-virus strains C57BL/6 and DBA/2.

In crosses with NFS, NZB mice were shown to have two unlinked dominant loci governing expression of infectious X-MuLV detected by focus formation in mink cells. Expression of infectious virus in NZB's differs in various lymphoid tissues: high levels of X-MuLV were found in bone marrow, somewhat lower levels in spleen and lymph node, and the lowest levels in thymus. Expression of infectious MuLV in T cells is influenced by genes governing their differentiation since T cells purified from spleen produce 10-fold more infectious X-MuLV than do thymocytes. Furthermore splenic B and T cells produce equivalent amounts of infectious virus.

Expression of X-MuLV-coded gp70, termed XenCSA, on the surface of lymphocytes was detected by flow microfluorometry using fluorescein-labeled rabbit antibodies specific for X-MuLV gp70. A survey of over 100 inbred strains for expression of XenCSA on their thymocytes and spleen cells revealed marked variations among these strains, but three basic phenotypes were observed: some strains have high antigen expression in spleen and thymus; others have low expression in thymus with high expression in spleen; and the great majority exhibit low antigen expression in both tissues.

Genetic crosses between a strain with high XenCSA levels (DBA/2) and one with low levels (C57BL/6) showed that XenCSA levels are determined by a semi-dominant gene on chromosome 4 in close proximity to Fv-1. Although this locus has the predominant effect on XenCSA levels, other, as yet undetermined factors act to modify the level of expression.

In crosses between NZB and NFS, it was shown that high XenCSA levels correlate with expression of only one of the two loci producing infectious X-MuLV and that a third locus may code for XenCSA expression in the absence of infectious virus.

Studies now in progress indicate that the patterns for X-MuLV expression (as infectious virus or XenCSA) in F/St mice do not follow the patterns established for NZB's.

Publications:

Chused, T. M., and Morse III, H. C.: Expression of XenCSA, a cell surface antigen related to the major glycoprotein (gp70) of xenotropic murine leukemia virus, by lymphocytes of inbred mouse strains. In Morse III, H.C. (Ed.): Origins of Inbred Mice. New York, Academic Press, 1978, pp. 297

Morse III, H. C., Chused, T. M., Boehm-Truitt, M., Mathieson, B. J., Sharrow, S. O., and Hartley, J. W.: XenCSA: Cell surface antigens related to the major glycoproteins (gp70) of xenotropic murine leukemia viruses. J. Immunol. 122: 443-454, 1979.

Morse III, H. C., Chused, T. M., Sharrow, S. O., and Hartley, J. W.: Variations in expression of xenotropic murine leukemia virus genomes in lymphoid tissues of NZB mice. J. Immunol. 122: 2345-2348, 1979.

Morse III, H. C., Chused, T. M., Hartley, J. W., Mathieson, B. J., Sharrow, S. O., and Taylor, B. A.: Expression of xenotropic murine leukemia viruses as cell-surface gp70 in genetic crosses between strains DBA/2 and C57BL/6. J. Exp. Med. 149: 1183-1196, 1979.

Morse III, H. C., and Chused, T. M.: Polymorphisms: Wild Mouse. Part II. Relationship to other retroviruses. In Altman, P. L. and Katz, D. D. (Eds.): Inbred and Genetically Defined Strains of Laboratory Animals. Part I. Mouse and Rat. Maryland, Federation of American Societies for Experimental Biology, 1979, pp. 229-232.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AI-00139-14 LMI

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

The Immunologic Response of Animals to Trypanosomal Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. F. Finerty	Research Microbiologist	LMI/NIAID
OTHER:	D. M. Dwyer	Research Microbiologist	LPD/NIAID
	L. P. Gasbarre*	Post-doctoral Fellow	
	L. Kendrick	Biologist	LMI/NIAID
	R. McKelvin**	Student	
	Y. Rosenberg	Visiting Scientist	LMI/NIAID
	E. Melvin***	Graduate Student	
	C. T.Hansen	Geneticist	DRS/NIH

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*** Laboratory Clinical Immunology, Med. Univ. of S. Carolina, Charleston, S. C.

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Laboratory of Microbial Immunity

SECTION

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INSTITUTE AND LOCATION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
18/12	10/12	8/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mice infected with various species of African trypanosomes, succumb 23-120 days after infection. Survival depends on the presence of IgM antibody. Mice with delayed IgM responses survive the longest. The data indicate a possible role of IgM antibody as a blocking agent.

PHS-6040
(Rev. 10-76)

Project Description:

The purpose of this study was to assay the host immune response in laboratory animals immunized or infected with African trypanosomes, the causative agents of African Sleeping Sickness. Previous studies indicated that animals could be actively immunized against Trypanosoma rhodesiense and that this protection was T cell dependent. The present course of investigation is focused on (1) isolation of the antigen(s) responsible for immunization and (2) the use of immuno-deficient animals to assay the humoral response.

Recently, methods were developed that can isolate and separate surface coat antigen, cytoplasmic organelles e.g. kinetoplasts and cell membranes from T. rhodesiense. A lysing solution, consisting of low ionic strength salts plus a chelating agent was developed that allows the isolation of the parasite into the components mentioned above. A gradient solution combined with centrifugation allows these components to be separated and isolated. These techniques will allow not only the immune response to these components to be analyzed but also the area of the cell biology of these parasites to be analyzed. Using SDS acrylamide electrophoresis, the cell membranes were resolved into major components that are similar to results obtained with other hemoflagelates. After completion of electron photomicrography, these results will be submitted for publication. This is the first time that membranes from the human pathogen, T. rhodesiense have been isolated.

The second aspect of this study concerned the humoral response to African trypanosomes utilizing naturally occurring immuno-deficient animals e.g. CBA/N and congenitally athymic (nude) mice. Recently, we found that "normal" mice i.e. normal immune responders succumbed to T. rhodesiense infections in 20-28 days after infection, whereas CBA/N mice survive longer than 50 days.

Since trypanosomes stimulate the host to synthesize large amounts of IgM and this was suggested to interfere with a protective host immune response we decided to study the effects of trypansomes in mice with a naturally occurring IgM antibody deficiency (T-independent) to polysaccharide antigens, and compare them to normal mice. Thus, CBA/N (IgM "deficient") were compared to CBA/CaJ (normal) mice upon infection with T. rhodesiense. The most obvious result was that CAB/CaJ mice and a mean survival of 23 ± 2 days, whereas CBA/N mice had a mean survival time of 62 ± 8 days after infection. Immunoglobulin (Ig) levels were quantitated in both strains of mice throughout infection. A characteristic pattern observed in CBA/CaJ mice was a rise in all Ig isotype levels, followed by a rapid decrease within 16 days after infection. CBA/N mice revealed similar Ig responses without the marked decrease observed in CBA/CaJ mice. The biggest difference was observed in the IgM antibody responses. IgM antibody was detected in CBA/CaJ mice during the 2 weeks following infection, and then became undetectable. In contrast, IgM antibody was undetectable in CBA/N mice for 14 days after infection, and then was detected on day 16, and thereafter. Congenic B6:CBA/N mice revealed a similar finding. The data suggest that a delay in the synthesis of IgM antibody is beneficial to hoast survival. Whether this reflects a delay in IgM blocking antibody to the trypansomes or a delay in other IgM antibody responses e.g. "autoimmune" antibody is under investigation.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AI-00140-14 LMI

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Immunology of Malaria

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. F. Finerty	Research Microbiologist	LMI/NIAID
OTHER:	L. Kendrick	Biologist	LMI/NIAID
	R. McKelvin	Student	LMI/NIAID
	W. P. Weidanz*	Professor	LMI/NIAID
	R. Rank**	Assistant Professor	LMI/NIAID
	D. Roberts***	Research Microbiologist	LMI/NIAID
	A. Tagliabue	Visiting Scientist	LIB/NCI

COOPERATING UNITS (if any)

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**University of Arkansas for Medical Sciences, Little Rock, Arkansas
***National Toxicologic Research Center, Little Rock, Arkansas

LAB/BRANCH

Laboratory of Microbial Immunity

SECTION

Microbiology and Immunology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
8/12	4/12	4/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX(c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Malarial antigen was used to both induce and elicit delayed hypersensitivity (DH) reactions in various strains of mice. Protection was correlated with DH in the early aspect of the immune response. This correlation appears to be related to macrophage activity.

PHS-6040
(Rev. 10-76)

Project Description:

The purpose of these studies is to assay the host immune response to rodent malarial parasites Plasmodium berghei, P. vinckei and P. yoelii. Previous studies demonstrated that T-cells were a necessary component in protection against lethal and non-lethal strains of rodent malaria. This was manifest in vivo by delayed-hypersensitivity reactions (DH) in mice by the footpad swelling technique. Swiss mice showed greater DH to malarial antigens than inbred strains of mice. Further, P.yoelii is a resolving infection in Swiss mice, but most inbred strains of mice become, or are susceptible to this parasite. This is particularly evident with BALB/c and C_3H mice. The latter were particularly susceptible to the non-lethal P. yoelii; death occurred 7-14 days after infection. Since the parasitemia reached >80%, this suggested that the organism changed when inoculated into C_3H mice. Parasitized C_3H blood was subsequently injected into Swiss mice and parasitemia was monitored. The parasites never exceeded 15%, same as the regular strain of P. yoelii, and no deaths occurred. This data suggested an inherent defect in the C_3H mice that made them unable to clear the parasites. DH responses to malarial antigen was less in C_3H mice than in Swiss mice. Macrophages are a necessary component in the host's ability to clear malarial parasites plus they are vital in DH responses. Preliminary results indicated that the responsiveness of C_3H macrophages to malarial parasites plays an important role in C_3H survival.

Futher studies will investigate and delineate the role of macrophages in resistance to malaria.

Publications:

Finerty, J. F., Kendrick, L. P. and McKelvin, R.: Chemical Enhancement of Protective Immunity. In Proceedings of a Food and Drug Administration Conference, Inadvertant Modification of the Immune Response, Washington, D. C., in press, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00141-05 LMI

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Immune Responses to Malaria and Related Intracellular Protozoa

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. W. Taylor	Staff Fellow	LMI/NIAID
	R. Asofsky	Laboratory Chief	LMI/NIAID
Other:	Y. Rosenberg	Visiting Fellow	LMI/NIAID
	K. J. Kim	Visiting Associate	LMI/NIAID
	P. E. McKeever	Research Associate	LMI/NIAID
	C. B. Evans	Biological Lab Tech	LMI/NIAID
	J. Rodrigues-Ramos	MBS Student	LMI/NIAID

COOPERATING UNITS (if any)

NONE

LAB/BRANCH

Laboratory of Microbial Immunity

SECTION

Microbiology and Immunology Section / Experimental Pathology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryalnd 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
32/12	24/12	8/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A biochemical and functional approach is being used to analyze the antigenic similarities and differences between the 17XL and 17XNL strains of Plasmodium yoelii. Over 125 malarial polypeptides have been identified using isoelectric focusing and SDS-PAGE. It appears that both quantitative and qualitative differences exist between the two strains. To date, twelve hybridoma cell lines, which secrete antiplasmodial antibodies (Ab), have been established. Monoclonal Ab are being used to identify and isolate important antigens of the erythrocytic stages of the parasite. In addition, polypeptides of P. yoelii are being separated by preparatory electrophoresis and fractions which contain antigens that react with hyperimmune serum and induce an in vitro proliferation response of sensitized, but not normal, lymphocytes have been identified.

PHS-6040
(Rev. 10-76)

Project Description:

A study has recently been initiated to evaluate the role of malarial antigens in immune protection and pathogenesis. Two strains of the rodent malaria parasite Plasmodium yoelii are being investigated. The 17XL strain routinely produces a fatal infection in BALB/c mice; whereas the 17XNL strain causes a self-limiting infection. Mice which have recovered from infection with the 17XNL strain are completely protected from challenge with the lethal form. Four basic research approaches are being used to isolate and identify protective and/or suppressive malarial antigens.

A. Biochemical Analysis of Plasmodial Polypeptides

A procedure has been developed for the isolation of intact erythrocytic stages of P. yoelii which results in a preparation of parasites relatively free of host material. Blood, collected from BALB/c mice with patent 17XL or 17XNL P. yoelii infections, is washed free of serum proteins and passed through a Whatman CF11 column to remove host leukocytes. Normal and parasitized erythrocytes are incubated in 0.65% NaCl for ten minutes resulting in swelling, but not lysis, of red blood cells (RBC). Parasites are released from swollen erythrocytes by the application of gentle pressure in a French pressure cell. Freed parasites and RBC ghosts are layered onto a discontinuous metrizamide gradient and centrifugated. Four distinct bands are produced. Fractions containing rings + merozoites, mature trophozoites and schizont-segmenters are collected washed and solubilized in the non-ionic detergent NP40. Such preparations provide the starting material for biochemical and immunologic (Section C) studies.

The technique of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze the above preparations of 17SL and 17XNL polypeptides. Gradient gels stained with Coomassie Blue revealed 8-10 bands (m.w. 25,000-300,000) with both quantitative and qualitative differences between the two strains. A new protein silver staining technique (100 times more sensitive than Coomassie Blue) has been used on reduced and non-reduced samples following SDS-PAGE resulting in the identification of 30-35 distinct bands. When parasite preparations were analyzed using 2-dimensional electrophoresis (proteins separated in the first dimension by isoelectric point differences using isoelectric focusing and by molecular weight differences in the second dimension using SDS-PAGE) at least 125 polypeptides were identified. Currently proteins of the 17XL and 17XNL strains and various stages of P. yoelii are being analyzed in this way. (Drs. Taylor, McKeever, Mr. Evans, and Mr. Rodriques-Ramos (student)).

B. Use of Monoclonal Antibody for Identification, Characterization and Isolation of Malarial Antigens

During the last five months a number of hybridomas have been produced which secrete anti-malarial antibody (Ab). These hybridomas were produced by fusing non-immunoglobulin secreting BALB/c myeloma cells (P3-X63-NS1/1) with

BALB/c spleen cells removed from mice infected with 17XL or 17 XNL P. yoelii four days prior to fusion. Fusions using spleen cells from hyperimmune mice (completely protected from challenge) which had been boosted four days earlier with P. yoelii have also been made. Following fusion, cells were cultured in microtiter wells and culture supernatants were screened for anti-plasmodial Ab using a solid phase radioimmunoassay (RIA). The assay consisted of binding freed parasites plus some RBC ghosts (see Section A) to poly-L-lysine coated polyvinyl u-shaped microtiter wells, blocking of excess charges with bovine serum albumin, applying test culture supernatants, and developing with an ^{125}I-polyvalent goat anti-mouse Ig. The isotype of the monoclonal anti-plasmodial Ab was determined using iodinated monospecific reagents. Positive hybrids were cloned and injected intraperitoneally into pristane-primed mice. Currently 12 hybridomas (seven from 17XL, four from 17XNL, and one from hyperimmune fusions) are being maintained and it is hoped they will prove to be stable cell lines.

Monoclonal Ab produced by eight of the hybridomas have been studied in detail. Three are secreting IgG_{2a} and the other five, IgM. One of the Ab (M4) binds to antigens (Ags) on the surface of freed parasites but the remainder recognize internalized Ags. All 12 of the monoclonal Abs bind to Ags common to all erythrocytic stages of the parasite (i.e. rings, trophozoites and merozoites), and thus do not demonstrate the presence of stage specific Ags. One of the four monoclonal hybrids (NLA10) produced by fusion with 17XNL spleen cells produces Ab which seems to recognize an Ag unique to the 17XNL strain (determined by indirect fluorescent Ab (IFA) staining and RIA analysis). All other hybridoma Ab appear to bind equally to both strains. An IFA study showed that some of the hybridoma Abs recognized only P. yoelii Ags, while some reacted with Ags shared among P. yoelii, P. berghei and Babesia microti.

Monoclonal Ab are currently being used to isolate parasite antigens employing the techniques of immune precipitation and affinity chromatography. Isolated antigens will be biochemically analyzed. (Drs. Kim, Taylor, and Mr. Evans).

C. Isolation and Identification of Immunologically Relevant Malarial Antigens

Preparations of parasite proteins are being fractionated by agarose preparatory electrophoresis (PE) and DE52 chromatography. Fractions are tested for the presence of malarial Ags by Ouchterlony analysis using hyperimmune BALB/c serum and for the ability to transform immune lymphocytes by an Ag-induced proliferation assay. PE fractions 5-8 and the third peak eluted from DE52 columns (0.05M buffer) have been shown to contain parasite antigens. Further biochemical fractionation of malarial polypeptides will be undertaken in the coming year and immunologically relevant Ags will be analyzed biochemically. (Dr. Taylor and Mr. Evans)

D. Identification of Antigens Associated with Autoimmune Hemolytic Anaemia

Inoculation of irradiated parasites (17XL strain) into BALB/c mice results in the induction of plasma cells secreting Ab against bromelain-treated normal mouse red blood cells (BrMRBC), an autoimmune phenomena. This observation suggests that the presence of parasite Ags or antigenic determinants on parasitized RBC is sufficient to produce an autoimmune response. Active infection is not required.

The anti-BrMRBC response also appears to correlate with lethality in that 17XL infections induce a specific anti-BrMRBC plaque forming cell (PFC) response peaking at day four, while the PFC response seen during the 17XNL infection occurs later and parallels the non-spcific B cell activation seen for the total Ig secreting cell populations. Serial passage of the 17XNL strain in BALB/c mice frequently results in the conversion of the parasite to the lethal form. The anti-BrMRBC response of the 17XNL strain closely approaches the 17XL kinetics with successive passages, thus probably reflecting antigenic changes in the parasite before increases in parasitemia and lethality are observed in the host.

Since 17XL and 17XNL strains infect mature RBC and reticulocytes respectively, it was investigated whether the cell type involved contributed to the different anti-BrMRBC response kinetics in the two infections. PFC assays using bromelain-treated reticulocytes or mature erythrocytes showed that anti-BrMRBC antibodies are predominantly directed to antigens on mature RBC indicating that only neoantigens expressed on the mature cells induce such a response and partially explain why specific anti-BrMRBC responses are not seen during stable 17XNL infections. Chemical characterization of such neoantigens are being undertaken and may yield information as to whether anti-BrMRBC Abs contribute to the anaemia or lethality which occurs following infection with the 17XL strain. (Dr. Rosenberg and Mr. Evans)

In addition to the above studies, several other areas of investigation are currently being undertaken to elucidate the mechanisms of immunity to intracellular protozoans.

Malarial Induced Immunosuppression

Immunization of mice, infected 8-11 days previously with 17XNL, with the antigens SRBC or TNP-ficoll induces no antibodies specific for these antigens. Such suppression has been shown to require T lymphocytes and macrophages. These experiments were repeated in malaria immune mice to study some of the conditions required to generate suppression. In such immune mice reinfection with malaria, subsequent immunization with SRBC yielded no suppression despite marked activation of T and B cells showing that activation of macrophages by large numbers of parasites is important in generating suppression and delineating the mechanisms responsible for polyclonal B cell activation from those causing suppression. (Dr. Rosenberg)

Studies on Resistance to Babesia Microti Infections

In experiments aimed to determine the role of T and B lymphocytes in infections with B. microti, mice suppressed for IgM production or control mice infected with this parasite and parasitaemias were followed both after primary infection and challenge. Such suppressed mice showed an unexpected resistance to B. microti infections compared to control mice and in addition were fully protected to reinfection. These studies suggest a role for IgM antibody in the invasion of erythrocytes by the babesial organism and may yield important information on mechanisms of invasion by intracellular parasites in general. (Dr. Rosenberg and Mr. Evans)

Publications:

Weinbaum, F. I., Weintraub, J., Nkrumah, F. K., Evans, C. B., Tigelaar, R. E., and Rosenberg, Y. J.: Immunity to Plasmodium berghei yoelii in mice. II. Specific and non-specific cellular and humoral responses during the course of infection. J. Immunol., 121: 629-636, 1978.

Rosenberg, Y. J., and Evans, C. B. Resistance of mice suppressed for IgM production to infection with Babesia microti. Nature, 1979 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00142-06 LMI

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Development of Thymus-Derived (T) Suppressor and Amplifier Cell Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. J. Baker	Head, Microbiology and Immunology Section	LMI/NIAID

COOPERATING UNITS (if any)

NONE

LAB/BRANCH
Laboratory of Microbial Immunity

SECTION
Microbiology and Immunology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0/12		

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Inactive during current year.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00143-10 LMI

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Genetic Control of the Antibody Response to Type III Pneumococcal Polysaccharide (SSS-III)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. J. Baker	Head, Microbiology & Immunology Sec.	LMI/NIAID
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	G. Caldes	Chemist	LMI/NIAID
	D. F. Amsbaugh	Biologist	LMI/NIAID
	P. W. Stashak	Microbiolgoist	LMI/NIAID
	D. W. Bailey**		

COOPERATING UNITS (if any)
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**The Jackson Laboratory, Bar Harbor, ME 04609

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Laboratory of Microbial Immunity

SECTION
Microbiology and Immunology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
20/12	6/12	14/12

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Congenic-resistant strains of mice, possessing chromosomal segments from a high-responding strain on a low-responding background strain, were used to isolate the genes involved in determining the capacity of bone marrow-derived precursors of antibody-forming cells (B cells) to make an antibody response to Type III pneumococcal polysaccharide (SSS-III) and to assign such genes to well-defined genetic linkage groups or chromosomes. The results obtained show that, although at least five genes influence the capacity of B cells to make an antibody response to SSS-III, these genes are located on different (more than three) chromosomes.

PHS-6040
(Rev. 10-76)

Project Description:

The results of previous studies have shown that the capacity of bone marrow-derived precursors of antibody-forming cells (B cells) to make an antibody response to Type III pneumococcal polysaccharide (SSS-III) is regulated by the activities of thymus-derived (T) suppressor and amplifier lymphocytes. Several autosomal dominant genes, acting in an independent manner, influence the functional activities of all three cell types and thus determine the magnitude of the antibody response ultimately produced after immunization. None of these functional activities were found to be linked to genes with the major histocompatibility (H-2) or the immunoglobulin allotype complex.

C57BL/6By mice produce an extremely low antibody response to SSS-III, α1,3 dextran and polyvinylpyrolidine (PVP), whereas BALB/cBy mice produce a high antibody response to these antigens. The availability of B6 congenic-resistant strains having chromosomal segments from high-responding BALB/cBy mice on a low-responding (C57Bl/6By) background provides a splendid opportunity to identify and isolate genes involved in determining responsiveness to SSS-III and to assign such genes to well-defined genetic linkage groups or chromosomes. To this end 18 of 23 available B6 congenic strains were immunized with an optimally immunogenic dose (0.5 μg) of SSS-III and the magnitude of the antibody response produced was assessed. The responses obtained were compared to that of low-responding C57BL/6By mice to determine whether the introduction of known chromosomal segments from BALB/cBy mice (high responders) caused a significant increase in the magnitude of the antibody response. Although 13 of 18 B6 congenic strains examined gave responses like those of low-responding C57BL/6By mice, five of 18 produced responses which were significantly greater than, but not equal to, that of high-responding BALB/cBy mice. In the latter case, segments from chromosomes 17, 9 and 4 as well as chromosomal segments containing the H-23 and H-27 loci appeared to contribute to the increased responsiveness noted. These findings suggest that genes governing the capacity of B cells to respond to SSS-III may be located on different chromosomes. But, it remains to be established whether the activities of these isolated genes are specific for the antibody response to SSS-III or influence the response to other antigens as well. Studies are in progress to resolve this issue and to determine if such genes act in a complementary (additive) manner.

Publications:

Rudbach, J. A. and Baker, P. J. (Eds.): Immunology of Bacterial Polysaccharides. Developments in Immunology, Volume 2. New York, Elsevier/North Holland, 1979, 157 pp.

Rudbach, J. A., and Baker, P. J.: Contributions of studies with bacterial polysaccharide antigens. In, Rudbach, J. A. and Baker, P. J. (Eds.): Immunology of Bacterial Polysaccharides. New York, Elsevier/North Holland, 1979, pp. 1-19.

Baker, P. J., and Prescott, B.: Regulation of the antibody response to pneumococcal polysaccharides by thymus-derived (T) cells: Mode of action of suppressor and amplifier T cells. In, Rudbach, J. A. and Baker, P. J. (Eds.): Immunology of Bacterial Polysaccharides. New York, Elsevier/North Holland, 1979, pp. 67-105.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00144-15 LMI

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Regulation of the Antibody Response to Type III Pneumococcal Polysaccharide (SSS-III).

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. J. Baker	Head, Microbiology & Immunology Sec.	LMI/NIAID
Other:	K. B. Schroer	Research Associate	LMI/NIAID
	K. J. Kim	Visiting Associate	LMI/NIAID
	P. W. Stashak	Microbiologist	LMI/NIAID
	D. F. Amsbaugh	Biologist	LMI/NIAID
	G. Caldes	Chemist	LMI/NIAID
	B. Prescott*		

COOPERATING UNITS (if any)

* Biological Research Institute, Rockville, MD 20852

LAB/BRANCH

Laboratory of Microbial Immunity

SECTION

Microbiology and Immunology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
20/12	14/12	6/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Somatic cell (lymphocyte-myeloma) hybrids from fusions of long-lived cell lines with antibody-forming cells from mice immunized with Type III pneumococcal polysaccharide (SSS-III) were used to investigate several basic issues associated with the antibody responseto SSS-III. The results obtained showed that the plaque-forming cell (PFC) repertoire expressed after immunization with an optimally immunogenic dose of SSS-III was reiterated in the distribution of isotypes found among hybrids making antibody specific for this antigen. Using the monoclonal products of hybridomas as probes to examine the diversity of the V-regions of antibody specific for SSS-III, it was found that the CC4.6 cross reactive idiotype is common and expressed on several classes of immunoglobulin with specificity for SSS-III.

PHS-6040
(Rev. 10-76)

Project Description:

Plasmacytoma cell lines which are drug marked, i.e., resistant to either 6-thioguanine or 8-azaguanine were fused in the presence of polyethylene glycol to spleen cells from mice immunized with Type III pneumococcal polysaccharide (SSS-III); here, the drug marked plasmacytoma cell line dies when cultured in medium containing hypoxanthine, aminopterin and thymidine (HAT). Subsequent cell growth is a feature of hybrid cells (hybridomas), protected from the lethal effects of HAT by the HGPRT enzyme from the X-chromosome of spleen cells from immunized mice. Clones secreting antibody specific for SSS-III can then be selected by limiting dilution assays. After sub-cloning in vitro, desired fusion products can be transferred to mice to elicit ascites and large amounts of monoclonal antibody specific for SSS-III; such antibody can be used to prepare anti-idiotype antibody in guinea pigs (or other animals) for use in V-region gene analysis.

For the antibody response to SSS-III, idiotypes present on hybridomas immunoglobulins were sought in sera from immunized mice. The first such screen of hybrids demonstrated the presence in sera (interstrain and intrastrain) of an idiotypic cross-reaction with a hybridoma immunologulin, designated CC4.6. This CC4.6 cross-reactive idiotype (CRI) appears to be present on a large proportion of the serum antibody specific for SSS-III as shown by similar inhibition profiles of immune serum and affinity purified CC4.6 immunoglobulin. No other hybrid product cross-reacted with the CC4.6 idiotype. Several lines of evidence indicate that CRIs elicited in hybridization experiments will reflect the repertoire expressed in immunized mice.

Each of the other hybrids making antibody specific for SSS-III produced unique idiotypes; these were not present in immune serum in detectable quantities. Since the plasmacytoma cell line used to generate these hybrids secreted IgG2b, κ immunoglobulin, one explanation for the absence of these idiotypes in immune serum is that the antiidiotype specificity is directed against a mixed molecule (parental myeloma κ chain + the H chain from immune spleen cells); but, the results of solid phase radioimmunoassays failed to confirm this possibility. Although antibody made by these clones may represent <30% of the serum antibody produced after immunization, subsequent hybridization failed to yield clones bearing these idiotypes which may occur in low frequency. Thus, although uncommon clones may be fused randomly, a large number of clonotypes must be examined to provide an accurate measure of their precursor frequency and diversity. In other hybridization experiments, the CRI was found to occur commonly and the isotype distribution of hybridoma immunoglobulins was similar to that for the antibody response of mice immunized with SSS-III (chiefly IgM). The CRI was common and expressed on several classes of immunoglobulin; in fact, the isotype distribution was similar to that found after immunization with SSS-III.

(CBA/N X BALB/c)F_1 male mice (CB mice) bear an X-linked defect making them unable to mount an antibody response to SSS-III. However, somatic cell hybrids between non-responding CB mice and plasmacytoma cells were found

to secrete IgM antibody specific for SSS-III. The solid phase binding of such antibody was completely inhibited by the addition of free antigen (SSS-III). The solid phase binding of such antibody was completely inhibited by the addition of free antigen (SSS-III) and the amount of antibody detected in culture fluids ranged from 10 ng/ml to 10 g/ml. Eight hybridoma clones were derived from such a fusion; all made antibody of the IgM class. These results indicate that the X-linked genetic defect does not result from the deletion of a B cell subset, capable of responding to certain thymus-independent antigens.

Publications:

Schroer, K. R., Kim, J. K., Amsbaugh, D. F., Stashak, P. W., and Baker, P.J.: Lymphocyte hybridomas which secrete antibodies to the Type III pneumococcal polysaccharide: idiotypic characterization. In, Schlessinger, D. (Ed.): Microbiology 1980, Washington, D.C., American Society for Microbiology, 1980, in press.

Schroer, K. R., Kim, J. K., Prescott, B., and Baker, P. J.: Generation of anti-Type III pneumococcal hybridomas from mice with an X-lined B-lymphocyte defect. J. Exp. Med., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00145-12 LMI

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (30 characters or less)
Mode of Action of Thymus-Derived (T) Suppressor and Amplifier Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. J. Baker	Head, Microbiology & Immunology Sec.	LMI/NIAID
Other:	K. B. Schroer	Research Associate	LMI/NIAID
	P. W. Stashak	Microbiologist	LMI/NIAID
	D. F. Amsbaugh	Biologist	LMI/NIAID
	G. Caldes	Chemist	LMI/NIAID
	B. Prescott*		

COOPERATING UNITS (if any)
*Biological Research Institute, Rockville, Maryland 20852

LAB/BRANCH
Laboratory of Microbial Immunity

SECTION
Microbiology and Immunology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
10/12	4/12	6/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Sheep erythrocytes sensitized with a hybridoma immunoglobulin capable of binding specifically with Type III pneumococcal polysaccharide (SSS-III) were used as indicator cells for the detection of plaque-forming cells (PFC) making anti-idiotypic antibody. Significant numbers of PFC making antibody against the CC4.6 idiotype, a common idiotype found in the serum of mice immunized with SSS-III, were detected during the late stages of an antibody response to an optimally immunogenic dose of SSS-III; larger numbers were found in mice given a high dose (tolerogenic) dose of SSS-III. The relationship of such antibody to the mode of action of thymus-derived suppressor and amplifier cells is being investigated.

PHS-6040
(Rev. 10-76)

Project Description:

There is ample evidence to indicate that the antibody response to Type III pneumococcal polysaccharide (SSS-III) is regulated by the activities of two functionally distinct types of thymus-derived (T) cells having opposing functions; these cells have been designated suppressor and amplifier T cells. Also, there is evidence to support the fact that the antibody response is regulated by a network of cellular interactions involving the formation of antibody and anti-idiotypic antibody; regulatory T cells have been implicated in such a network. Therefore, it was of interest to determine whether anti-idiotypic antibody is produced during the course of an antibody response to SSS-III and whether the development of such antibody is associated with the expression of suppressor T cell activity.

The development of somatic cell hybrids secreting large amounts of antibody specific for SSS-III and bearing a common cross-reactive idiotype (see Z01 AI 00144-15 LMI) enables one to examine this issue at the cellular level. Here, erythrocytes sensitized with SSS-III-binding hybridoma immunoglobulin can be used as indicator cells is the technique of localized hemolysis-in-gel for the detection of plaque-forming cells (PFC) making anti-idiotypic antibody. The results of preliminary studies show that significant numbers of PFC making anti-idiotype antibody were found in mice late after immunization with an optimally immunogenic dose of SSS-III; however, greater numbers were detected in mice given a large (tolerogenic) dose of antigen. Very few - if any - PFC making anti-idiotypic antibody were detected after priming with a marginally immunogenic dose of SSS-III, a procedure known to activate suppressor T cells. Although it would be tempting to speculate that suppressor T cells are involved in the formation of anti-idiotypic antibody, such antibody could provide another independent mechanism for the regulation of the antibody response. In this context, suppressor T cells have been shown to act mainly by limiting the extent to which B cells proliferate following immunization; but, anti-idiotypic antibody may limit the magnitude of the antibody response by inhibiting the secretion of antibody by antigen-stimulated B cells as has been suggested by the work of other investigators. These issues are now being investigated.

Publications:

Pasanen, V. J., Asofsky, R., and Baker, P. J.: Synthesis of two classes of antibody, γM and γG or γM and γA by identical cells. Amplification of the antibody respone to pneumococcal polysaccharide Type III. J. Exp. Med., 149: 1227-1237, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00146-06 LMI

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Immunological Studies on Components Isolated from Bacteria, Parasites and Plants.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. J. Baker	Head, Microbiology & Immunology Sec.	LMI/NIAID
Other:	B. Prescott*		
	G. Caldes	Chemist	LMI/NIAID
	D. F. Amsbaugh	Biologist	LMI/NIAID
	P. W. Stashak	Microbiolgoist	LMI/NIAID
	P. R. Beining**		
	G. M. Flannery**		

COOPERATING UNITS (if any)

*Biological Research Institute, Rockville, Maryland 20852
**Department of Biology, University of Scranton, Scranton, Pennsylvania 18510

LAB/BRANCH

Laboratory of Microbial Immunity

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Microbiology and Immunology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
12/12	2/12	10/12

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The IgM antibody response to the lipoteichoic acid (LTA) isolated from staphylococcus aureus ATCC 6538P was examined by a newly developed procedure in which erythrocytes, sensitized with periodate-activated LTA, were used for the detection of IgM-producing plaque-forming cells (PFC). LTA-specific PFC were first detected two days after immunization with heat-killed bacteria and maximal PFC were attained by day four; specificity of such PFC was affirmed by plaque-inhibition tests. No PFC were found in mice given isolated LTA over a 10,000-fold range of immunizing doses. Mice, pretreated with a carrier known to activate thymus-derived (T) helper lymphocytes, produced a PFC response to LTA only when immunized with LTA bound to the same carrier. This suggests that carrier-specific helper T cells are needed to initiate an antibody response to poorly-immunogenic LTA. Since an antibody response can be elicited in mice given heat-killed bacterial cells, bacterial cell wall and/or cell membrane components may play an important role as immunological carriers of this molecule.

PHS-6040
(Rev. 10-76)

Project Description:

The lipoteichoic acid (LTA) of gram positive bacteria is an antigen well-suited for use in basic research on various immune phenomena. It is composed of a glycerol phosphate polymer, covalently linked to a lipid moiety, and contains glycosyl and D-alanyl side groups. LTA is known to interact with host-membrane and such interactions have been implicated in the development of arthritis. LTA may also serve as a carrier of immunocytotoxic antigens or, as an exposed antigen, may play a decisive role in the binding (colonization) of bacteria to epithelial cells of mucosal surfaces. Although the latter suggests that antibodies specific for LTA may play an important role in limiting the pathogenesis of staphylococcal infections, this has been difficult to investigate; isolated LTA is considered to be a poor immunogen in experimental animals, mainly because it has been found to generate extremely low serum antibody and antibody-producing plaque-forming cell (PFC) responses after immunization. Such apparently low antibody responses may be due to the insensitivity of methods commonly used for the detection of serum antibody and antibody-forming cells.

We have developed a method for the sensitization of indicator erythrocytes using LTA that has been activated by treatment with periodate. Erythrocytes sensitized by this method are stable and can be used effectively not only in conventional passive immune hemolysis and hemagglutination tests for the detection of small amounts of serum antibody, but also in the technique of localized hemolysis-in-gel for the detection of cells making antibody specific for LTA. The results obtained using such indicator erythrocytes were both reproducible and specific for LTA, the immunizing antigen.

Although mice were found to give a reasonably good serum antibody and PFC response specific for LTA after immunization with heat-killed Staphylococcus aureus, little or no antibody could be detected in mice given isolated (purified) LTA over a 10,000-fold range of immunizing doses. Prior treatment with a relatively large dose of isolated LTA did not reduce the manitude of the antibody response produced to an optimally immunogenic dose of heat-killed S. aureus, indicating the LTA is not tolerogenic. However, mice pretreated with a carrier known to activate thymus-derived (T) helper lymphocytes, enabled mice to mount an antibody response to LTA, only when immunized with LTA bound to the same carrier; the magnitude of the antibody response produced was at least as great as that made after immunization with an optimal dose of heat-killed S. aureus, even though the total amount of carrier-bound LTA used was extremely small (non-immunogenic in mice not pretreated with carrier). These findings suggest that carrier-specific helper T cells are needed to elicit an antibody response to poorly-immunogenic LTA; since an antibody response can be produced in mice immunized with heat-killed S. aureus, bacterial cell wall and/or cell membrane constituents must play an important role in this regard.

The availability of a sensitive and reproducible method for the detection of antibody specific for LTA now permits us to characterize the class (isotype)

of antibody produced in response to LTA and to examine the role of such antibody in the development of protective immunity to staphylococci. Also, studies with carrier molecules, more appropriate for use in man, are in progress to enhance the immunogenicity of isolated (purified) LTA for potential use in vaccine studies.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00153-03 LMI

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
In Vitro Response of Human Peripheral Lymphocytes to Infectious Organisms

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Richard Asofsky	Laboratory Chief	LMI/NIAID

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Microbial Immunity

SECTION
Experimental Pathology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0/12		

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

No work was done on this project this year.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00158-03 LMI

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
The Immune Response to Entamoeba Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. F. Finerty Research Microbiologist LMI/NIAID

COOPERATING UNITS (if any)
NONE

LAB/BRANCH
Laboratory of Microbial Immunity

SECTION
Microbiology and Immunology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0/12		

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Inactive during current year.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00186-06 LMI Formerly Z01 DE 0084-05

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Pathogenesis of Autoimmunity in Inbred Strains of Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. M. Chused	Medical Officer (Internal Med)	LMI/NIAID
OTHER:	H. C. Morse, III	Senior Investigator	LMI/NIAID
	W. Davidson	Visiting Fellow	LMI/NIAID
	J. Longstreth	NIH Post-doctoral Fellow	LMI/NIAID
	S. O. Sharrow	Chemist	IB/NCI
	E. Brown	Microbiologist	LMI/NIAID

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Microbial Immunity

SECTION
Experimental Pathology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
26/12	16/12	10/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to understand the pathogenesis of autoimmune disease by studying murine modesl such as New Zealand Mice. The present areas of investigation are the early activiation of the immune system, manifested by macrophage activation and immunoglobulin production; B and T cell membrane receptors and differentiation antigen abnormalities; and the regulation of xenotropic murine leukemia virus production and virus coded cell surface antigens. The genetic analysis of these parameters has identified several separate genes which presumably contribute to the development of autoimmune disease.

PHS-6040
(Rev. 10-76)

Project Description:

The spontaneous autoimmune disease of New Zealand Black (NZB) mice has been under investigation for more than 15 years without a definitive explanation for their disorder. We have previously shown that NZB splenic B cells spontaneously produce 40 to 100 times as much pentameric IgM as non-autoimmune mice.

Further investigation of NZB B cells, much of it utilizing the fluorescence activated cell sorter, has shown:

1. NZB spleen contains a greatly increased number of IgD-dull cells.

2. The IgD-dull cells include a subpopulation of large, IgM secreting plasmablasts.

3. These plasmablasts are polyclonally activated.

4. NZB spleen contains ten times as many such plasmablasts as normal mice.

5. Each plasmablasts contains and secretes four to five times more IgM than those of normal mice.

6. Ablation of T cells by thymectomy, irradiation and reconstitution with anti Thy 1 treated bone marrow or by introducing the nude gene onto the NZB background does not prevent the spontaneous B cell activation.

We also observed that NZB T cells contained pinocytozed IgM, presumably anti-T cell autoantibody. To determine whether this process accounts for NZB T-cell abnormalities, such as their ability to give a primary in vitro cytotoxic response to minor histocompatability antigens of H-2 compatible mice, NZB B cell function was suppressed with anti-IgM antibody. Such mu-suppressed mice gave the same level cytotoxic response to minor histocompatibility antigens, indicating that NZB mice also have a primary T cell defect. A macrophage abnormality that could produce both the T and B hyperfunction has not been ruled out.

Genetic analysis of both the T and B abnormalities is in progress.

Dr. Wendy Davidson has examined "motheaten" mice, a spontaneous, recessive mutation on chromosome 6 in C57BL/6J mice. Motheaten B cell express charges similar to, but more extreme than, those of NZB cells. Their T cells also appear to be blast-like.

Preliminary study of BXSB and MRL mice indicates that they have patterns of immunologic activation which differ from those of NZB and motheaten. Thus, we now have several distinct models of murine autoimmune disease whose investigation should prove fruitfull.

In the course of our NZB studies we have shown that they carry at least two and perhaps three separate xenotropic murine leukemia virus genes. These lines are now in the seventh to ninth backcross. We are now examining the relationship of these viruses to autoimmune disease.

To facilitate the use at the two-color capability, we developed in collaboration with Research Organics, Inc. of Cleveland, Ohio, a modified rhodamine-like fluorophore, XRITC, which has absorption and emission maxima 28 nm higher than those of tetramethylrhodamine isothiocyanate.

Publications:

Chused, T. M., Moutsopoulos, H. M., Sharrow, S. O., Hansen, C. T. and Morse, H. C. III: Mechanism of autoimmune disease in New Zealand Black mice. In Rose, N. R., Bigazzi, P. E., and Warner, N. L., (Eds.): Genetic Control of Autoimmune Disease. Elsevier North-Holland, New York. (In press), 1979.

Davidson, W. F., Morse, H.C. III, Sharrow, S. O., and Chused, T. M.: Phenotypic and functional effects of the motheaten gene on murine T and B lymphocytes. J. Immunol., 122: 884-891, 1979.

Chused, T. M., Moutsopoulos, H. M., Sharrow, S. O., and Hanson, C. T.: Evidence of a primary B lymphocyte abnormality in NZB mice. In Cooper, M., Mosier, D., Scher, F., and Vitetta, E., (Eds.): B Lymphocytes in the Immune Response, Elsevier North-Holland, New York. (In press), 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00187-06 LMI Formerly Z01 DE 0085-05

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Studies in Sjogren's Syndrome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. M. Chused	Medical Officer	LMI/NIAID
OTHER:	E. M. Brown	Biologist	LMI/NIAID
	H. Moutsopoulos	Visiting Scientist	LMI/NIDR
	T. Lawley	Senior Investigator	D/NCI
	D. Mann	Senior Investigator	I/NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Microbial Immunity

SECTION

Experimental Pathology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4/12	2/12	2/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to determine the mechanism and etiology of the autoimmune disease Sjogren's Syndrome. Genetic factors, such as HLA-A, -B and -D type and Ia-like B cell autoantigens are of particular interest. In addition, clinical parameters such as circulating immune complexes are evaluated.

PHS-6040
(Rev. 10-76)

Project Description:

We have previously shown that Sjogren's syndrome (SS) is associated with the serologically defined histocompatible antigen HLA-B8 (50% + in SS, 21% + in controls) and, to a greater extent, with the lymhpocyte defined antigen HLA-Dw3 (84% + in SS alone, 24% + in controls).

These associations were studied in greater detail by typing a series of 24 SS patients with a panel of 60 alloantisera directed against human Ia antigens. Two antisera, Ia-172 and Ia-AGS, reacted with 100% of SS patients but 25 and 61% of normals, respectively. These antigens are not associated in the normal population. This suggested that two Ia antigens and thus possibly two immune response genes may be required for the development of SS.

We recently expanded our series of SS patients and separated them into those with SS alone (primary) and SS with reheumatoid arthritis (secondary). Interestingly, we observed HLA antigen differences between the two groups. Patients with primary SS tended to have three independent "families" of HLA antigens: (1) HLA-B8 and HLA-DRw3 (2) Ia-172, Ia-350 and (3) Ia-715. Patients with secondary SS tend to carry (1) HLA-DRw4 (known to be associated with rheumatoid arthritis) and (2) Ia-172 and Ia-350.

From this we conclude that the Ia-172 complex may facilitate the development of both primary and secondary SS; that primary SS, in fact, requires three immune responses genes; and that the immunostimulation associated with rheumatoid arthritis may abrogate the requirement for HLA-DRw3 and Ia-715 observed in the primary disorder. It is worth noting that Ia-715 is found in both primary SS and systemic lupus erythematosus two diseases which antinuclear antibodies are frequently found.

The genetic differences between primary and secondary SS prompted a retrospective chart review of these patients. This study showed that salivary gland enlargement, splenomegaly and several other clinical manifestations are all significantly more frequent in primary SS.

In collaboration with Dr. Thomas Lawley we investigated circulating immune complexes in SS. We found them to be very common and correlated with clinical activity of the disease. Because serum rheumatoid factor is also very frequent in SS we carefully demonstrated that the immune complexes are not primarily composed of rheumatoid factor and immunoglobulin.

Many case reports have indicated that lymphoma occurs relatively often in SS. We undertook an epidemiologic study to qualify this risk, finding it to be 44 times that of the normal population. Waldenstom's macroglobulin is also much more frequent in SS.

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Moutsopoulos, H. M., Balow, J. E., Lawley, T. J., Stahl, N. I., Antonovych, T. T. and Chused, T. M.: Immune complex glomerulonephritis in sicca syndrome. American J. Med. 64:955, 1978.

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Moutsopoulos, H. M., Webber, B. L., Vlagopoulos, T. P., Chused, T. M., and Decker, J. L.: Differences in the clinical manifestations of sicca syndrome in the presence and absence of rheumatoid arthritis. Amer. Jour. of Med. 66:733-736, 1979.

LABORATORY OF PARASITIC DISEASES

1979 Annual Report

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Laboratory of Parasitic Diseases

National Institute of Allergy and Infectious Diseases

SUMMARY - October 1, 1978 through September 30, 1979

INTRODUCTION

Over the past year many changes at various levels of professional personnel occurred. Carl Mattern, M.D., who had previously been in the Laboratory of Viral Diseases, was transferred to LPD because of administrative changes in LVD, but also because Dr. Mattern's research interests have included protozoology and viruses of amebae. Gabriel Schmunis, M.D. who was a Visiting Fellow with us some years ago and now works in Brazil, joined us as a Visiting Scientist. Senior Staff Fellow Charles Oster, M.D. left us to take a senior infectious disease position at Walter Reed Medical Center. Phillip Smith, M.D., a gastroenterologist from the University of Colorado, is due to join Dr. Nash late in the year as an IPA employee for work on giardiasis. Fouad Boctor, Ph.D., a Visiting Associate from Egypt, will be returning there late this fiscal year, and T. Takeuchi, Ph.D., of Japan, another Visiting Associate who worked with Dr. Weinbach, returned to Japan at the beginning of this reporting year. Two Research Associates, Brian Catto, M.D. and Thomas Quinn, M.D. finished their two year tours with us and left to take further post-doctoral work in Cleveland and Seattle, respectively, while one new Associate, Mark Hofstetter, M.D. just arrived. Three new Visiting Fellows who joined us were Maria do Carmo de Souza, Ph.D. of Brazil, D.C. Kaushal, Ph.D. of India and Nuzhat Anwar, Ph.D. of India. Renato Gusmao, M.D. of Brazil who had been with us as a Visiting Fellow, started graduate work at Johns Hopkins School of Hygiene, but retains an appointment as Guest Worker with us to participate in field research on Chagas' disease in Brazil. Another Guest Worker from Brazil, M. Barral, M.D. who has a Kellogg Foundation Fellowship arrived to work with Dr. Cheever. Dr. Martinez-Paloma of Mexico spent one month with Dr. Diamond as a Guest Worker in July. The move of Dr. Richards and Dr. Sullivan to a contract facility in Rockville for their research on snail vectors of schistosomiasis was delayed but will probably take place in late summer or early fall.

Participation in field research abroad continued with Dr. Ottesen and Ms. Stanley working on filariasis for several weeks in Madras, India and Drs. Neva and Gusmao, along with Mr. Gam and several of our collaborators from Duke University working again in Brazil on Chagas' disease. Dr. Neva returned to Brazil for follow-up on the collaborative project in Goiania, as well as to collect triatome vector bugs of Chagas' disease in Paulo Afonso. Drs. Gwadz and Beach of the Malaria Section spent several weeks in Egypt as advisors on a PL-480 project and Dr. Rosenberg spent several weeks as an advisor to AID in Bangladesh, where he had worked previously. Drs. Ottesen and Nash are scheduled to visit and consult with CDC personnel in Puerto Rico about possible collaborative work on schistosomiasis.

Laboratory of Parasitic Diseases

National Institute of Allergy and Infectious Diseases

SUMMARY - October 1, 1978 through September 30, 1979

RESEARCH ACCOMPLISHMENTS

MALARIA RESEARCH PROGRAM OF THE LPD, NIAID

Search for antigens with hybridomas: The new technique of fusing spleen cells from immunized mice with myeloma cells is being used to identify specific antigens of malaria parasites. Polyethylene glycol is used to promote fusion of cells and the resulting hybrid cells are grown in a selective medium which permits growth only of hybrid cells. When the resulting hybrid cells are cloned, the resulting progeny, or "hybridoma" cells produce a monoclonal antibody *in vitro* or *in vivo* as an ascites tumor in mice, which is characteristic of myeloma cells. One objective of this technique is to use the monoclonal antibody produced by the hybridoma to identify specific components of malaria parasites. Both internal and surface antigens of gametes of *P. gallinaceum* have been identified with this method (Rener, Rosenberg of LMI, Carter and Miller). Hybridoma cell lines have also been developed that react with surface components of merozoites of *P. knowlesi*, as determined by fluorescent antibody reactions against living intact merozoites. (Epstein, Miller and Carter).

Gamete physiology and immunology: One of the major remaining mysteries about the malarial parasite is how development of gametocytes is controlled. Are some of the asexual forms destined to become gametocytes or are there external stimuli which initiates their development? With the *in vitro* culture system of Trager and Jensen the conclusion has been reached that environmental stimuli control gametocyte production of *P. falciparum.* (Carter and Miller). Also, the addition of cyclic AMP to the culture medium was found to stimulate conversion of asexual forms to gametocytes (Kaushal, Carter and Miller).

Surface antigens of merozoites of *P. knowlesi* were found to be shared by antigens of *P. falciparum*. (Miller, Johnson and Carter). The common antigenic specificity between these two species of malarial parasites might have some future practical application. Further work has been done to try to characterize the so-called exflagellation factor, a material in the gut of mosquitoes which induces gametogenesis in gametocytes of *P. gallinaceum.* By sephadex chromatography the substance appears to be of low molecular weight (only 200 to 300), it is stable to heat and contains several amino acids. (Beach).

Merozoite receptor: Although evidence for a receptor substance on red blood cells which permitted invasion of certain species of malaria parasites was established previously, the exact immunochemical nature of the receptor has not yet been identified. Another aspect of the merozoite-red cell interaction is the properties of the merozoite which involve attachment to

red cells. Mild treatment of merozoites of P. knowlesi with trypsin was found to destroy ability of the merozoite to invade red cells. Such treatment was associated with loss of certain high molecular weight bands on SDS polyacrylamide gel electrophoresis, suggesting that these proteins may be involved in attachment of merozoites to red cells. (Johnson, Epstein, Shiroishi and Miller.

Sporozoite antigens: Collaborative work with Dr. Nussenzweig at NYU is aimed at a more precise understanding of immunity to sporozoites of P. knowlesi in the rhesus monkey. Immunity to sporozoite challenge could be transferred passively and was consistently associated with demonstration of anti-sporozoite antibodies by various in vitro tests. Attempts are being made to identify the antigen(s) responsible for protection by ^{125}I labelling and by hybridoma production of specific antibodies. (Gwadz and Nussenzweig of NYU).

Anti-malarial drugs affecting gametocytes and liver forms: It has been noted that anti-malarial drugs such as primaquine that affect exo-erythrocytic liver forms generally also inhibit gametocyte development in the mosquito. Since a system for testing oocyst development in mosquitoes fed upon P. gallinaceum infected chickens is readily available and the same effect can also be checked by a membrane feeding system, these two types of tests are being used to check drugs for activity against exo-erythrocytic stages. Thus far, a good correlation was found for drugs having gametocytocidal effects and their ability to act as hepatic schizontocides. (Gwadz, Koontz, Miller and collaborators at WRAIR).

Anopheline physiology: The steroid hormone, ecdysone, until recently believed absent in adult mosquitoes, has been shown to play a major role in oogenesis. Recent studies have shown that ecdysone also modulated feeding behavior and blood-meal retention in female mosquitoes and mating behavior in males. Genetic analysis of vector competence of an anopheline vector in Egypt, A. pharoensis, has been initiated in an effort to identify factors determining susceptibility. (Beach and Rosenberg).

Role of the spleen in defense against malaria: Last year it was reported that the role of the spleen in host defense against malaria seemed to depend more upon rheologic than immunologic factors. This conclusion was confirmed by studies of clearance in the rat of ^{51}Cr-labeled red cells parasitized with P. berghei. Even though merozoite invasion of RBCs could be prevented in vivo by antibody, acute resolution of malaria was spleen dependent and involved decreased deformability of infected erythrocytes and altered splenic micro-circulation. (Quinn and Wyler). Congenitally asplenic mice, as well as adult splenectomized mice, could not overcome an acute malarial infection. Reconstitution of asplenic mice with syngeneic or autologous spleen cells did not restore protective function. (Wyler and Oster).

CELL BIOLOGY AND PHYSIOLOGY OF PROTOZOAN PARASITES

Amebae: The mechanism of action and partial characterization of a "toxin" from extracts of E. histolytica has been achieved. Con A, a plant lectin, was found to have a nearly identical effect as the amebal toxin on cell cultures. The effects of the amebal toxin can be inhibited by fetuin, a serum glycoprotein, and the amebal toxin and Con A are inhibited by certain hexosamines; therefore, lectin-like properties seem to be involved. (Mattern). Amebae of greatly increased virulence, as judged by the ability to produce liver abscesses in the newborn hamster, were selected by passage in the newborn hamster, whereas virulence is usually lost by passage in axenic medium only.(Mattern). There have been some additional improvements in the axenic cultivation technique for E. histolytica. Yeast extract as one of the essential components has been replaced with a mixture of defined ingredients consisting of purines, pyrimidines, nucleosides and B complex vitamins. Use of butyric acid has been found to be a key factor in a method devised to permit direct axenization of E. histolytica from mixed bacterial cultures. (Diamond). Investigation of the sequence of electron flow in respiration of axenized E. histolytica is now quite complete. It goes from nicotinamide adenine nucleotides, flavoproteins, only small amounts of quinones and terminating in iron-sulfur centers. Since the iron-sulfur centers are one-electron transfer carriers the end product of their reduction is H_2O rather than H_2O_2 which would be the end product of flavins as has been claimed by others. (Weinbach and Claggett).

Giardia: A considerable range of studies has been initiated with Giardia lamblia. Maximal growth, resistance to oxygen and attachment to glass surfaces was found to be dependent upon the presence of L-cysteine. Serum or plasma fractions also promoted attachment of giardia trophozoites to glass surfaces. In contrast, secretory immunoglobulins inhibited attachment. Factors involved in excystation of cysts are also under study. In spite of this accumulation of knowledge about giardia attempts to initiate new cultures from patients have been unsuccessful. (Gillin). Biochemical studies have also been initiated with giardia, since it can be grown axenically in medium developed for amebae. It is similar to E. histolytica in that it has no mitochondria, cytochromes or a tricarboxylic cycle, but in contrast its respiratory enzymes are particulate and has a different type of non-heme iron proteins. (Weinbach, Diamond and Keister).

Leishmania: The time spent on perfecting techniques to isolate membranes and membrane constituents of leishmanial parasites has proven to be rewarding. First of all, good yields and clean preparations by electromicroscopy have been obtained of pellicular membranes and microtubules of L. donovani. By use of lectin-fluorescein conjugates, components of the pellicular membrane could be "stained", indicating that the membranes have at least 20 or so glycoprotein constituents with various side chain carbohydrates, as well as an actin or actin-like constituent. (Dwyer). Antibodies produced in rabbits against these pellicular membranes have disclosed evidence of polysaccharide antigens common to 6 different trypanosomatid species. (Gottlieb and Dwyer). Also, this common antigen was detected as an exoantigen in the sera of L. donovani infected hamsters. (Dwyer). Freeze-fracture and-etching studies

by EM have demonstrated many details of supramolecular structure of the pellicular membranes. (Da Silva and Dwyer). Study of membrane enzymes, especially acid phosphatase, has shown that the flagellate form of the organism is already prepared for life within the host cell phagolysosomes! (Gottlieb and Dwyer). The kinetoplast-mitochondrial fraction of the organisms is being studied for its respiratory metabolism, a point of interest because most biochemical work on leishmanial kinetoplasts has focused mainly on their DNA. (Weinbach and Dwyer). The techniques used for study of leishmanial membranes are being applied to isolation and characterization of membranes of Trypanosoma rhodesiense. (Finerty and Dwyer). The human peripheral monocyte which becomes a macrophage in culture is being used as a host cell for L. donovani and L. tropica to investigate the leishmanial-host cell interactions that permit growth of the parasite within phagolysosomes. (Berman, Dwyer and Wyler). The BALB/c mouse inoculated in the foot-pad so that resulting lesions can be assessed more precisely to characterize virulence has given quite a range of responses with different strains of leishmania from cutaneous lesions. (Neva).

Trypanosoma cruzi: The factor(s) responsible for enhanced attachment to and penetration of cells by trypomastigotes that is present in the serum of chronically infected humans or animals appear to be in the $\geq$50,000 molecular weight fraction of serum. IgG or IgM fractions separately were inactive and absorption with protein-A decreased the activity. (Schmunis and Dvorak). DNA synthesis in intracellular T. cruzi was found to undergo a synchronous cycle after a pre-replicative lag of at least 12 hours. It was concluded that T. cruzi trypomastigotes are in the G_0-G_1 phase of their cell division cycle and that parasite reproduction occurs independently of events controlling the host-cell DNA synthesis cycle. These findings of synchronous growth of amastigotes should facilitate biochemical studies on this phase of the parasite. (Crane and Dvorak). One of the handicaps to the procedure of producing hybrids from vertebrate cells X T cruzi epimastigotes has been a more efficient method of selecting definite hybrid clones. Use of a different vertebrate cell, one which lacks certain nutrients and can therefore be selected for by using selective medium, should help obviate this problem. The P3-X63Ag8 cell line seems to serve this purpose. (Crane and Dvorak).

The plaquing technique for clonal selection of intracellular T. cruzi has now been tested sufficiently to be applicable to study of parasite strains directly from infected bugs. Concomitant titration of the same trypomastigote suspension for infectivity at serial dilutions and for plaque formation indicates that the minimal infective dose, or ID_{50}, is not necessarily equivalent to plaque forming units. Therefore, the property of producing plaques requires more than the ability to simply initiate infection of cells. Ability of strains to produce plaques is better at 33°c, in fact, very few strains have been found to produce plaques at elevated temperature. In this respect, growth curves and plaque formation give similar results. (Neva). An interesting phenomenon of bacterial-parasite interaction which came to notice accidentally has been investigated. Filtrates of Pseudomonas fluorescens were found to produce morphologic changes in trypomastigotes of T. cruzi, including lysis. The main effect of the bacterial product appears to be on the cell membrane, and the lytic

factor is resistant to heat, is not inhibited by trypsin and has a molecular weight ~10,000 daltons. (Mercado).

HELMINTHIC INFECTIONS

Immune response in human schistosomiasis: Antibodies to a glycoprotein fraction from adult schistosome worms when measured by the ELISA procedure were found to correlate with intensity of infection. (Nash and Lunde). The levels of antibody in patients at various time stages of their infection were related to antigens from appropriate stages of the parasite - e.g. higher levels to cercarial antigen in acute disease and higher levels to adult worm antigen in chronic cases. Although IgE to schistosome antigens can be measured by the ELISA technique, it is not as sensitive as conventional procedures. (Lunde and Ottesen).

Schistosomal antigens and neurotransmitters: After labelling in vitro cultured schistosomes with radioactive amino sugars excretory-secretory (E-S) materials have been studied. Addition of various metabolic inhibitors to the system can either increase or decrease the release of large and small molecular weight substances into the medium. This approach is being used to study synthesis and turnover of schistosome tegument as well as antigenic E-S components of the parasite. (Nash). The kinetics of uptake and metabolic pathways leading to synthesis of serotonin, a major neurotransmitter of schistosomes, have been defined in the schistosomule which is the early developmental stage. Previous work on serotonin was with adult worms. (Catto).

Experimental pathogenesis of schistosomiasis: Collagen content of the liver in rabbits with Symmers' fibrosis due to S. japonicum infection increased greatly up to 30 weeks after infection. The liver pathology did not continue to progress beyond this time because of death of worms and decreased oviposition of remaining worms. Markedly different intestinal lesions in rabbits were found to be produced by a Japanese as contrasted to a Phillipine strain of S. japonicum. The Phillipine strain produced serosal tumor-like masses and hence egg excretion did not reflect intensity of infection. It will be important to determine whether such differences in oviposition exist in human infections. (Cheever). The substance from schistosomal egg granulomas which was found to stimulate collagen synthesis and fibroblast proliferation of mammalian cells was found to be distinct from soluble egg antigen, and was found to also increase c-AMP and prostaglandin E 2 synthesis in cell cultures. (Wyler, Wahl, Cheever and Wahl).

Snail vectors of schistosomiasis: Now a total of as many as 12 genetically different susceptibility types of Biomphalaria glabrata snails to S. mansoni have been demonstrated. Another mechanism for insusceptibile snails has been found in which the miracidia penetrate but fail to develop despite an apparent absence of host tissue reaction. This type of insusceptibility is being referred to as "insuitability" of the vector snail, in contrast to the "resistant" snails in which miracidia are encapsulated and destroyed by amebocytes. This "resistant" type of insusceptibility can be reversed by preinfection of the snail with a heterologous trematode parasite, such as Echinostoma paraensei, but the "unsuitable" type of insusceptibility is not altered by heterologous preinfection. (Richards).

Three different genetic stocks of B. glabrata are being tested for their ability to develop resistance of the 4 most commonly used molluscicides. Successive generations of survivors of exposures to calculated 90 percent lethal doses are being reared for tests of possible molluscidide resistance. (Sullivan).

Host response to filarial infection: Patients with tropical pulmonary eosinophilia who have no circulating microfilarial (mf) because they are efficiently trapped in the lungs represent one extreme of the human response to filarial infection. At the other extreme are those who are asymptomatic and circulate abundant mf. This hypo-responsiveness appears to involve serum inhibitory factors. These latter patients may also have specific cellular immune hypo-responsiveness which appears related to both serum and suppressor cell factors. (Ottesen). Complement-enhanced leukocyte adherence has been demonstrated with mf and immune serum. Chromium-labelled mf of D. immitis are being studied for more precise information of their clearance in immune animals. (Weil and Ottesen).

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HONORS AND AWARDS

Dr. Louis H. Miller continues to serve on the editorial boards of Experimental Parasitology, Journal of Parasitology and Journal of Molecular Medicine. He is a member of the Steering Committee for Immunology of Malaria of the TDR of WHO, and was elected to membership in the American Society for Clinical Investigation.

Dr. Louis S. Diamond was appointed a member of the Executive Committee of the Society of Protozoologists and serves on the editorial board of Experimental Parasitology.

Dr. Mattern serves on the editorial board of the Journal of Protozoology.

Dr. Cheever is a member of the editorial board of the American Journal of Tropical Medicine and Hygiene.

Dr. Dwyer serves on the editorial board of the Journal of Protozoology and was re-appointed as adjunct Associate Professor at Rockefeller University and at the University of Massachusetts.

Dr. Neva completed several years of service on the editorial board of the Journal of Infectious Diseases and continues as a member of the editorial board of the American Journal of Tropical Medicine and Hygiene. He was re-appointed Visiting Lecturer at the Harvard School of Public Health and the Johns Hopkins School of Hygiene and Public Health. He is a member of the Steering Committee of the NAS-Institute of Medicine's study on Clinical Investigation in Developing Countries.

Dr. Ottesen served as Chairman of the NIAID Clinical Research Committee and began to serve on the editorial board of Experimental Parasitology. He was an invited participant in a WHO Immunology Course given in Lausanne, Switzerland and has been asked to serve as a member of the WHO TDR Scientific Working Group on Immunology of Filariasis. He was also elected to the Infectious Disease Society of America.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00091-17 LPD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

The Genetics, Biology, and Control of Snail Intermediate Hosts of Schistosomes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: C. S. Richards Parasitologist, LPD, NIAID

Other: J. T. Sullivan Visiting Fellow, LPD, NIAID

COOPERATING UNITS (if any) Dr. K. J. Lie and Dr. Donald Heyneman, The George Williams Hooper Foundation, University of CA., Dr. Ernesto Ruiz, San Juan Laboratories, Bureau of Laboratories, CDC, San Juan, P.R. Dr. David Woodruff, Dr. Phil LoVerde, Ms. Madeleine Fletcher, and Mr. Dennis Minchella, Dept. of Biology,

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Host-Parasite Relations Section

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20205

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CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Genetic variations occur in susceptibility to infection in Biomphalaria glabrata and infectivity in Schistosoma mansoni. Studies indicate at least 12 genetically different susceptibility types in B. glabrata. Thirteen strains or substrains of S. mansoni apparently differing genetically in snail infectivity are being compared. Ten of these are of Puerto Rican origin.

Two different mechanisms of insusceptibility to S. mansoni in various genetic stocks of B. glabrata are being compared: active resistance with amebocytic encapsulation and destruction, and unsuitability for parasite development in the absence of observed host tissue reaction. Genetic tendency in B. glabrata for amebocytic accumulations, and relationship between this and insusceptibility to infection with S. mansoni are being studied.

B. glabrata, when infected with x-irradiated miracidia of certain species of trematodes, acquires specific resistance to a subsequent challenge with nonirradiated miracidia of the same species. In addition, preinfection with

Cooperating Units (continued):

Purdue Univ., Dr. M. R. Kasschau, Dept. of Physics, M.D. Anderson Hospital and Tumor Inst., University of Texas.

Summary (continued):

nonschistosome miracidia interfers with the active resistance of the snails to S. mansoni, rendering them temporarily susceptible. These phenomena are being studied with the trematodes Echinostoma paraensei and Ribieroa marini.

The ability of three stocks of B. glabrata to develop resistance to the 4 most commonly used molluscicides is being compared. Successive generations of survivors of molluscicide exposures are being reared and tested for molluscicide resistance.

Project Description:

The objective is to study molluscan and trematode genetics with particular attention to applications to control measures. To study genetic variations in snail susceptibility and parasite infectivity. To study host-parasite interaction, including molluscan cellular defence mechanisms and unsuitability for parasite development. To study snail amebocytes particularly as related to defence against parasites. To study prospective agents for biological control of intermediate hosts of schistosomes. To study causes of tumors in mollusks. To study the biology of the snails with the aim of improving the efficiency of control measures. To study the genetics of molluscicide resistance in snails. To study the contributions and complications of molluscan diseases and molluscan genetics to biomedical research.

Occurrence in B. glabrata of two different pigmentation genes on different chromosomes has facilitated formal genetic studies. Each gene has three alleles (wildtype, blackeye, and albino; coalesced mantle pigment, discrete mantle spots, and absence of black mantle pigment) with combinations resulting in nine phenotypes. Six morphological single gene characters in B. glabrata have now been described, apparently all on different chromosomes. Crosses will be performed to determine if linkage occurs between any of these morphological characters and factors regulating susceptibility to S. mansoni infection. Attempted matings between each of three stocks of B. glabrata from different localities in Puerto Rico and ten genetically different laboratory stocks of B. glabrata, have indicated general incompatibility. Hybrid F_1s have resulted with only two of the laboratory stocks.

Need for evaluation of the role of genetics in snail control continues. Resistant B. glabrata may prove useful in biological control of schistosomiasis by dilution or replacement of populations of susceptible snails in endemic areas. Resistant B. straminea may be effective in interspecific

competition. Genetic resistant and susceptible stocks of B. glabrata have been provided Mr. Dennis Minchella, Purdue University, for laboratory competition studies. Progress in understanding causes of susceptibility or insusceptibility of B. glabrata to infection with S. mansoni accelerates the prospect of safe field testing of resistant snail stocks for control.

Individual snails of various B. glabrata stocks are exposed as juveniles, young adults, and old adults to miracidia of a series of S. mansoni strains. Selection is carried out until snail lines demonstrating consistent susceptibility or insusceptibility patterns are established. Snails of genetically characterized stocks are exposed to miracidia of various S. mansoni strains and substrains, with selection until consistent infectivity patterns are characterized. Results indicate about 12 genetically different susceptibility types in B. glabrata; about 13 genetically different infectivity strains in S. mansoni. Ten of the S. mansoni strains or substrains are of Puerto Rican origin, involving collaboration with the San Juan Laboratories, CDC, P.R. (Dr. Ernesto Ruiz). Snail crosses and parasite crosses are in progress or planned to determine the genetic factors involved. Establishing genetic B. glabrata and S. mansoni lines provides material for biochemical, biological and cytogenetic intraspecific analyses. Such genetic lines have been provided on request to many other investigators. In collaborative studies at Purdue University, Dr. David Woodruff and Ms. Madeleine Fletcher are pursuing allozyme studies on our genetic lines of B. glabrata and S. mansoni.

Studies on the mechanisms involved in snail trematode interactions are continuing. When a free swimming S. mansoni miracidium in freshwater penetrates a potential host snail, it encounters a drastic osmotic change in the snail hemolymph. Ability to adapt to this change may involve the free amino acids of parasite and host. A former LPD guest worker, Dr. Margaret Kasschau (Univ. of Texas) studied the free amino acids of cercariae and adults of S. mansoni. Collaborative studies are planned to compare the free amino acids of B. glabrata hemolymph and S. mansoni of genetically characterized lines.

Two basically different mechanisms of insusceptibility to S. mansoni occur in host-parasite combinations involving various genetic lines of B. glabrata and S. mansoni. Active resistance involves host recognition with rapid amebocytic encapsulation and destruction. Unsuitability involves delayed or aborted parasite development in the absence of observed host tissue reaction. The occurrence of these phenomena is being surveyed in various B. glabrata-S. mansoni combinations, and snail crosses are in progress to study the genetics of these different host-parasite relations.

Two nonschistosome trematodes that infect B. glabrata are now cultured in our laboratory: Echinostoma paraensei and Ribieroa marini. With these parasites we are extending the studies of Lie and his coworkers, who have demonstrated acquired resistance to trematodes in B. glabrata. Snails sensitized by infection with x-irradiated R. marini miracidia acquire resistance to a challenge with nonirradiated R. marini miracidia

given 7 to 10 days subsequently. This resistance is acquired as soon as 3 days, and persists for at least 3 weeks post-exposure to irradiated miracidia. This marks the first independent confirmation of acquired resistance in molluscans and is the first demonstration of this phenomenon outside of the Echinostomatidae. Initial attempts to interfere with insusceptibility to S. mansoni (by first infecting insusceptible snails with irradiated E. paraensei and then challenging with nonirradiated S. mansoni) have indicated that this procedure will block insusceptibility that is due to active resistance, but not that due to unsuitability.

Amebocytes play an important role in defence of B. glabrata against infection by S. mansoni. Studies on occurrence of genetic amebocytic accumulations are continuing. In some genetic lines of B. glabrata transitory amebocytic accumulations occur in the atrium in young adults, during the period of maximum reproductive activity. When exposed to S. mansoni, these snails test susceptible as juveniles, insusceptible as young adults with atrial amebocytic accumulations, and revert to susceptibility in old age. Ultramicroscopic studies on these amebocytic accumulations are planned in collaboration with Purdue University (Dr. Phil LoVerde).

Microsporidia, amebae, and bacteria cause extensive damage in some snail hosts. Search for viral or fungal infections are also in progress. These and other snail diseases need to be examined for potential human infection, and prospective use as agents in biological control of snails. It is essential to be aware of the occurrence of these infections and their relations with the snails and other parasites to plan, conduct, and interpret experimental research on snails with validity.

Studies on a variety of tumors observed in B. glabrata, many of which are genetic, will be continued.

An inbred laboratory stock of B. glabrata, the M line albino, and two stocks recently sent to us from field collections, one from Puerto Rico and the other from St. Lucia, are being tested for resistance to the molluscicides, Bayluscide, Frescon, potassium pentachlorophenate, and copper sulfate. Successive isolation of and rearing of offspring from survivors of molluscicide exposures over several generations should result in some degree of resistance. This resistance will be characterized statistically with the use of a potency probit analysis computer program, and the genetics of its transmission will be studied. Currently, dosage-mortality regression plots, e.e., LC_{50} determinations, are being established for the 4 molluscicides in each of the 3 nonselected parental stocks.

Publications:

1. Kassim, O. O. and Richards, C. S.: Schistosoma mansoni: Lysozyme activity in Biomphalaria glabrata during infection with two strains of the parasite. Exp. Parasit., 46: 213-217, 1978.

2. Kassim, O. O. and Richards, C. S.: Biomphalaria glabrata: Lysozyme activities in the hemolymph, digestive gland and headfoot of the intermediate host of Schistosoma mansoni. Exp. Parasit., 46: 218-224, 1978.

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4. Kassim, O. O. and Richards, C. S.: Host reactions in Biomphalaria glabrata to Schistosoma mansoni miracidia, involving variations in parasite strains, numbers and sequence of exposures. Internat. Jour. for Parasit. (in press, 1979).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00092-13 LPD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
The pathogenesis of schistosome infections in mammalian hosts.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. W. Cheever	Assistant Chief, LPD, NIAID
Other:	T. E. Nash	Medical Officer, LPD, NIAID
	E. A. Ottesen	Medical Officer, LPD, NIAID
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	M. N. Lunde	Research Zoologist, LPD, NIAID

COOPERATING UNITS (if any) Southwest Foundation for Research & Education, San Antonio, TX (Dr. Kuntz); Dept. Gastroenterol. WRAIR (Dr. Dunn); Lab. Statistical and Math. Methodology, DCRT, NIH (Dr. Mosimann, Mrs. Minker); Div. Immuno-parasitology, NMRI (Dr. Dean)

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Host Parasite Relations

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.0	1.5

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Schistosome infections are studied quantitatively in man and experimental animals. Schistosoma japonicum infections in rabbits regularly produced Symmers' clay pipestem fibrosis of the liver, but this was often accompanied by a mixed macronodular-micronodular cirrhsis. Morphologic fibrosis and collagen content decreased between 30 and 50 weeks of infection. The size of hepatic granulomas around eggs remained nearly constant, but several factors combined to drastically reduce the number of eggs reaching the liver. Hepatic fibrosis was similar in rabbits infected with Japanese and Philippine strains of the parasite. The passage of eggs in the feces parallelled the intensity of infection in rabbits infected with the Japanese, but not the Philippine, worm strain, apparently because most eggs were not deposited near mucosal surfaces by the latter.

PHS-6040
(Rev. 10-76)

The nature and pathogenesis of lesions produced in mammalian hosts by schistosomes of medical importance are studied in man and experimental animals. Schistosome infections are quantitated by measurement of worm numbers, passage of eggs in excreta and accumulation of eggs in host tissues.

Symmers' fibrosis of the liver was produced in rabbits with heavy S. japonicum infection. Hepatic fibrosis, evaluated histologically and by measurement of hydroxyproline content of the liver, decreased markedly between 30 and 50 weeks after infection, confirming earlier microscopic findings of Japanese investigators. Several factors combine to decrease the insult to the liver during this time: 1) worm numbers decrease slightly 2) the number of eggs laid by each worm pair decreased by about 50% and 3) the proportion of eggs laid which reach the liver decreased markedly. Modulation of granuloma size was minimal, although this is an important factor in moderating pathology in several host species infected with S. mansoni. Cirrhosis in infected rabbits appears to be caused by the S. japonicum infection, a situation without a counterpart in man or other experimental animals. This is a major drawback to this model; but it remains the only experimental model, other than the chimpanzee, of Symmers' fibrosis of the liver.

Passage of eggs in the feces of rabbits infected with a Japanese strain of S. japonicum is proportion to worm numbers, but this is not the case with a Philippine strain, in which many less eggs are passed. This appears to be explained by focal deposition of eggs in the intestinal serosa by the Philippine strain. A few rabbits infected with the Japanese strain and showing similar anatomic lesions had similar patterns of egg passage. Passage of eggs in the feces is the only currently available technique for measuring intensity of schistosome infection in man, so that if generally valid this finding has clinical importance. We are therefore comparing the two worm strains in capuchin monkeys to see if this pattern holds for primates. Collagen synthesis in the liver and cell mediated immune responses are also being examined in the monkeys in collaboration with Dr. Ottesen (LPD) and Dr. Dunn (WRAIR).

Schistosome infections appear to produce much more fibrosis in the liver than in the gut, although egg deposition is similar in the two organs. We are pursuing this at the descriptive level and quantitating intestinal fibrosis through measurement of hydroxyproline levels.

The study of bladder "cancers" produced by S. haematobium in capuchin monkeys is nearly terminated. The papillary lesions of the bladder epithelium regressed with time (2 to 5 years after infection) and are probably inflammatory rather than neoplastic in nature. Regression of the lesions occurred as the schistosome infection disappeared. The monkeys had considerable resistance to reinfection and active infections of high intensity could not be maintained.

During the next year the reversibility of hepatic fibrosis in S. japonicum infected rabbits will be examined following chemotherapy, with or without subsequent colchicine treatment (Dr. Dunn). The biology, pathology and immunology of S. japonicum and S. mansoni infections in capuchin monkeys will be studied. Pathology and collagen metabolism in inbred strains of mice will be looked at in relation to their resistance to schistosome infection (Dr. Dean, NMRI and Dr. Dunn).

Understanding of the lesions caused by schistosomiasis in man, the associated morbidity and their relation to intensity of infection are basic to evaluating the importance of the disease and to some aspects of control and treatment. The detailed study of animal models contributes to our understanding of the parasite and its possible interactions with the human host.

1. Ottesen, E. A., Hiatt, R. A., Cheever, A. W., Sotomayor, Z. R. and Neva, F. A.: The acquisition and loss of antigen-specific cellular immune responsiveness in human schistosomiasis. Clin. Exp. Immunol. 33: 38-47, 1978.

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4. Cheever, A. W.: Schistosomiasis and neoplasia. J. Nat. Cancer Inst. 61: 13-18, 1978.

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6. Kuntz, R. E., Cheever, A. W., Bryan, G. T., Moore, J. A. and Huang, T. C.: Schistosomiasis: Natural history of papillary lesions in the urinary bladder in schistosomiasis. Cancer Research 38: 3836-3839, 1978.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00093-04 LPD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Studies of the Immunologic Response to Helminth Infections

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. A. Ottesen	Senior Investigator, LPD, NIAID
Other:	B. A. Catto	Research Associate, LPD, NIAID
	G. J. Weil	Research Associate, LPD, NIAID
	M. N. Lunde	Research Zoologist, LPD, NIAID
	A. W. Cheever	Assistant Chief, LPD, NIAID
	F. A. Neva	Chief, LPD, NIAID
	T. J. Lawley	Medical Investigator, NCI

COOPERATING UNITS (if any) Tuberculosis Chemotherapy Center, Madras, India (Dr. S.P. Tripathy); Medical College of Madras, India (Prof. K.V. Thiruvengadam). Biomedical Research Institute, Rockville, Md. (Dr. F.A. Lewis), Bureau of Veterinary Research, FDA, Beltsville, Md. (Dr. K.G. Powers).

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Host-Parasite Relations

INSTITUTE AND LOCATION
NIAID, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
62/12	38/12	24/12

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☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major goals of this project are to characterize the host's immune response to helminth infections and to relate the findings to the pathogenesis of clinical disease. Chronic filariasis and schistosomiasis are both characterized by cellular immune hyporesponsiveness to parasite antigens which may play a role in the persistence of the parasite within the host. The mechanisms involved in this hyporesponsiveness include both serum inhibitory factors and mononuclear suppressor cell populations. Profound immunologic hyperresponsiveness of the immediate type (IgE) characterizes the asthma-like tropical eosinophilia syndrome of human filariasis. The most important antigens are those derived from microfilariae. By contrast patients with patent microfilaremia are hyporesponsive. Clearance of these microfilariae depends on anti-surface antibodies. In acute schistosomiasis not only is there marked cellular and humoral immune activity but also the presence of large numbers of circulating immune complexes which may be important in the syndrome's pathogenesis.

Larval schistosomes (schistosomules) contain the biogenic amine, serotonin. Its uptake mechanisms and metabolic precursors have been defined.

Project Description:

The character and evolution of the immune response in man and experimental animals has been studied during and following infection with parasitic helminths.

Filariasis. The questions of why some individuals in an endemic area become infected with filaria (bancrofti) while others appear to resist it and of why even among infected individuals the clinical manifestations of disease can be so varied have led to a search for the relevant immunologic parameters both in field studies of human filariasis and in work on laboratory models of the disease. Field studies in the Cook Islands (Project #JO233-I-74) and in India (Project #76-I-231) have shown that in endemic areas the greatest cellular immune reactivity (lymphocyte transformation) and the highest production of antibodies to filarial antigens (determined with ELISA techniques) occur not in infected patients but in those equally exposed to infective parasites but who have not acquired the infection. In fact, there exists in infected individuals a state of specific cellular immune unresponsiveness to filarial antigens which appears to be determined both by serum inhibitory factors and by suppressor cells which can be identified among the adherent subpopulation of mononuclear peripheral blood cells. The nature of these suppressive elements is currently being explored. (Ottesen) Also being studied is the pathogenesis of the recurrent attacks of lymphangitis (i.e., filarial fevers) and the possible role of immune complexes in initiating them (Ottesen, Lawley).

Tropical eosinophilia (TE) is a form of filariasis characterized by chronic lung disease with episodes of paroxysmal nocturnal asthma, profound blood eosinophilia, markedly elevated IgE levels and high filarial antibody titers. Using the in vitro correlate of IgE-mediated allergic responses (i.e., the histamine release reaction of IgE-coated basophils challenged in vitro with the sensitizing allergen) and a radioenzymatic assay for the detection of histamine, we have documented and quantified the high degree of allergic sensitization to filaria in patients with TE (compared to those with other forms of filarial disease). The especial hypersensitization to microfilariae and their products may be most important in determining the symptoms and pathology associated with this syndrome (Ottesen, Neva). At the other extreme, individuals with circulating microfilariae who might be expected also to be allergically sensitized were paradoxically hyporesponsive to antigens derived from microfilariae. The mechanisms underlying this hyporesponsiveness appear to involve serum inhibitory factors whose nature is under study (Ottesen).

Microfilaria (MF) have been used as a model for the study of immunity to tissue helminths. In the canine and human systems most infected individuals have antibody directed against soluble microfilarial antigens. Individuals who are immune to the microfilarial stage (infected but amicrofilaremic) have circulating antibody directed against microfilarial surface structures. In the presence of immune serum normal peripheral blood leukocytes adhere to MF

in vitro. Cell adherance is enhanced by the presence of complement and accompanied by lysosomal enzyme release onto the surface of the MF (Weil, Ottesen).

Experiments involving the infusion of radiolabeled MF of Dirofilaria immitis into dogs have shown that injected MF circulate freely in normal and microfilaremic infected dogs, with relative concentration in the microvasculature. MF-immune dogs clear injected MF from the blood within fifteen minutes. The MF are primarily trapped in the lung (Weil, Powers, Ottesen).

Schistosomiasis. Previous studies in our laboratory have defined the progressive loss of parasite antigen-specific cellular immune responsiveness (lymphocyte transformation) in patients whose schistosome infections evolve from the acute to chronic stages. As we have explored the mechanisms involved in this modulation of antigen responsiveness, it has become clear that just as in the patients with filariasis, individuals with chronic S. mansoni infections have inhibitory elements both in their serum and in their adherent mononuclear cell populations. The serum and cell suppressive effects may be additive and their natures are being investigated (Ottesen).

Serum samples from patients followed closely for almost two years after developing acute schistosomiasis have been studied for the presence of $C1_q$ binding immune complexes. All patients with symptomatic acute schistosomiasis had circulating immune complexes which either declined gradually as the infection became chronic or declined rapidly after treatment. The finding of circulating complexes in patients with chronic infection was much less common (2 of 11 patients) than it was in acutely infected individuals. Studies to determine the antigenic make-up of these complexes and their potential role in the pathogenesis of the acute syndrome are in progress (Lawley, Ottesen).

Biochemical studies on the major neurotransmitter of schistosomes (i.e., serotonin) have defined the presence of this amine in the schistosomule stage of the organism. The kinetics of uptake and matabolic pathways leading to its synthesis have been defined (Catto). Cercariae of S. mansoni upon penetration of the skin release a factor causing discharge of amine-rich granules from tissue mast cells. This factor which is not found in the post-penetration schistosomules is likely responsible for the schistosome dermatitis which develops after initial exposure to infective schistosomes (Catto, Lewis, Ottesen).

Publications:

1. Ottesen, E. A., Hiatt, R. A., Cheever, A. W., Sotomayor, and Neva, F. A.: The Acquisition and Loss of Antigen-Specific Cellular Immune Responsiveness in Acute and Chronic Schistosomiasis in Man. Clin. Exp. Immunol. 33: 38-47, 1978.

2. Ottesen, E. A. and Weller, P. F.: Eosinophilia Following Treatment of Patients with Schistosomiasis mansoni and Bancroft's filariasis. J. Inf. Dis. 139: 343-347, 1979.

3. Ottesen, E. A., Neva, F. A., Paranjape, R. S., Tripathy, S. P., Thiruvengadam, K. V. and Beaven, M. A.: Specific Allergic Sensitization to Filarial Antigens in Tropical Eosinophilia Syndrome. Lancet I: 1158-1161, 1979.

4. Nash, T. E., Ottesen, E. A., and Cheever, A. W.: Antibody Response to a Polysaccharide Antigen Present in the Schistosome Gut. II. Modulation of Antibody Response. Am. J. Trop. Med. Hyg. 27: 944-950, 1978.

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14. Lunde, M. N. and Ottesen, E. A.: Enzyme-linked immunosorbent assay (ELISA) for detecting IgM and IgE antibodies in human schistosomiasis. Am. J. Trop. Med. Hyg. (In press).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00094-20 LPD

PERIOD COVERED
September 30, 1978 to October 1, 1979

TITLE OF PROJECT (80 characters or less)
Studies of Entamoeba histolytica and other Parasitic Protozoa

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	D. B. Keister	Biologist, LPD, NIAID
	E. C. Weinbach	Head, Physiology and Biochemistry Section, LPD, NIAID
	C. F. T. Mattern	Medical Officer, LPD, NIAID

COOPERATING UNITS (if any)
American Type Culture Collection, Rockville, MD; Hospital General, Centro Medico Nacional, I.M.S.S., Mexico City.

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Parasite Growth and Differentiation

INSTITUTE AND LOCATION
NIAID, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	1.3	2.7

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Yeast extract an ingredient of TYI-S-33 medium devised for axenic cultivation of Entamoeba histolytica has been replaced with a mixture of defined nutrients consisting of adenine, guanine, cytosine, uracil, AMP, ADP, ATP, and NH_4Cl fortified with B vitamins. A method has been devised enabling rapid direct axenization of E. histolytica from cultures in which the parasite is grown with a mixed bacterial flora and thus circumvents the intermediate step of growing the amebae monoxenically prior to axenization. Butyric acid added to the medium used for axenization is a key factor in success of this technique. An improved medium for axenic cultivation of Giardia lamblia has been achieved by means of supplementing TYI-S-33 medium with crude bovine bile preparations containing free and conjugated bile acids. Growth is superior to that attained with TP-S-1 currently the medium of choice for this parasite. Modification of a technique devised in this laboratory has enabled cyropreservation of refractory strains of E. histolytica. Success is attributed to the substitution of sucrose for glucose in the cryopreservant, the use of TYI-S-33 for cultivation of the amabae before and after freezing and the use of plastic vials in the place of glass.

Project Description:

The objectives of this project are 5 fold: 1) to develop and refine techniques for the axenic cultivation of Entamoeba histolytica, related Entamoeba and other parasitic protozoa; 2) to determine their nutritional requirements in vitro and to define the physical and chemical conditions for optimal growth; 3) to study the mechanisms of their pathogenicity; 4) to study and characterize the viruses of E. histolytica; 5) to devise or improve existing methods for the freeze-preservation of these parasites at cryogenic temperatures and to study mechanisms involved in resistance to freeze-preservation.

1. Development of a Defined Medium for Axenic Cultivation of E. histolytica - There are 3 undefined components of TYI-S-33 the medium currently used in our laboratory for axenic cultivation: Trypticase (casein digest), bovine serum and yeast extract (YE). Current investigations are aimed at replacing YE with defined nutrients. A highly purified (98%) commerical preparation of yeast mucleic acids (YNA) was found to support amebic growth provided it was supplemented with the equivalent concentrations of the B vitamins present in yeast. A mixture of adenine, guanine, cytosine, uracil, AMP, ADP and ATP in the proportions present in yeast supported serial subculture (25 to date) when substituted for YNA. The yields of amebae although considerably lower than those obtained with YNA were equal to the best yields obtained with the older TP-S-1 medium. Glutamic acid, glutamine and NH_4Cl when added separately to the above mixture of purines, pyrimidines and nucleosides further enhanced growth increasingly in the order presented. Addition of cholesterol and lecithin alone or in combination, or a standard mixture of trace metals did not enhance growth. Subtle effects could have been masked by the bovine serum and Trypticase which are rich sources of these nutrients.

Projected future studies entail determination of the optimal concentrations of the defined nutrients shown to be of value in replacement of YNA, testing of additional defined components of yeast, and attempts to replace trypticase and bovine serum with defined nutrients (Diamond and Cunnick).

2. Iron and Nutrition of E. histolytica - Diamond (1978) postulated that the protozoa, none of which have been shown to produce siderophores, could conceivably use preformed siderophores synthesized by other microorganisms in the environment as do certain bacteria and algae. In TYI-S-33 there are two sources of iron, that which occurs in the three biological ingredients and had been designated intrinsic iron, and that which is present in the ferric ammonium citrate (FAC) supplement, extrinsic iron. When established cultures of E. histolytica growing in TYI-S-33 were deprived of extrinsic iron through deletion of FAC, the rate of growth decreased and stabilized after a few subcultures at a lower rate. If in addition the intrinsic iron was chelated with Desferal, a potent microbial siderophore, growth was marginal. However, if FAC was combined with sufficient Desferal to bind all iron and was added to the medium in which the intrinsic iron was previously bound with Desferal, growth of the amebae was significantly increased. This increase was below that obtained with medium containing intrinsic iron alone, but well above that ob-

tained with the Desferal bound intrinsic iron. This suggests that the ameba can remove some iron from the Desferal complexes in vitro and could possibly meet some of its iron requirements in vivo through use of preformed microbial siderophores (Diamond and Keister).

Direct axenization of E. histolytica from Mixed Bacterial Cultures - A method has been developed enabling rapid direct axenization of E. histolytica from cultures in which the parasite in grown in the presence of a mixed intestinal flora (the customary culture technique for isolating the organism from the host) and circumvents the laborious intermediate step of growing the amebae monoxenically is association with a trypanosomatid or Fusobacterium symbiosus. Key factors in this technique are as follows: 1) Use of a newly devised liquid medium for growing the amebae with bacteria before axenization. The medium is specially designed to match TYI-S-33 ingredients and tonicity in order to facilitate transfer of the amebae from xenic to axenic conditions of growth. 2) Supplementation of TYI-S-33 with butyric and isobutyric acids during the axenization phase. We have noted that bacterial flora which produce butyric acid significantly enhance growth of several species of Entamoeba. Recently Scheff (1978) reported that the ability of Fusobacterium (see above) and allied species to produce butyric acid was related to their ability to support monoxenic growth of the E. histolytica-like Laredo strain of ameba. Attempts will be made to axenize other entamoebae, specifically E. coli and E. gingivalis. Neither of these species from man have been axenized (Diamond and Keister).

Improved Medium for Axenic Cultivation of Giardia lamblia - Studies originating in this laboratory have shown that although TYI-S-33 medium is far superior to TP-S-1 for axenic cultivation of E. histolytica, the reverse is true for G. lamblia. Except for the incorporation of yeast extract in the former medium and Panmede, an ox-liver digest, in the latter, the two media are essentially similar in respect to the other ingredients. The association of G. lamblia with the gall bladder in human giardiasis, the presence of bile constituents in Panmede, and the observation that partial substitution of Panmede for the yeast extract in TYI-S-33 improved its ability to support growth of G. lamblia led to exploration of the use of bile in the cultivation of this parasite. TYI-S-33 was supplemented singly with the following commercial preparations: 1) curde bile containing taurocholic acid (40%), glyocholic, cholic and deoxycholic acids in lower condentrations; 2) dehydrated unfractionated bovine bile, and 3) bacteriological grade of bovine bile consisting of a mixture of free and conjugated bile acids. Growth of G. lamblia in the presence of each of these supplements was markedly superior to that obtained with TP-S-1. The fact that a synthetic taurocholic acid of high purity (98%) failed to stimulate growth suggests that enhancement of growth by the crude preparations was not due alone to this particular compound. Addition of a quantity of Panmede to TYI-S-33 equal in weight to each of the above supplements was not sufficient to stimulate growth. This suggested that multiple factors in Panmede were responsible for its growth stimulating effect, or that the growth enhancing factors were present in low concentrations. Future studies will attempt to define the factors in bile which are responsible for stimulation of growth. Specific fractions commercially available or

prepared in the laboratory will be studied. Combinations of conjugated and free bile acids in varying proportions will be tested (Keister and Diamond).

Cryopreservation of E. histolytica - Modification of a technique originated in this laboratory has led to the cryopreservation of amebal strains which have been refractory in previous repeated trials. Success is attributed principally to having 1) substituted sucrose for glucose as one of the constitutents of the cryopreservant employed, 2) the use of TYI-S-33 instead of TP-S-1 for cultivation of the amebae prior to freeze-preservation, and as the basic freezing and recovery vehicle, 3) the use of plastic vials in place of glass. The following specific attributes of TYI-S-33 have been found to play vital roles in the success of freeze-preservation. Cells in late logarithmic or stationery phase are generally most suitable for cryopreservation. The relatively long logarithmic and stationery phases which are characteristic of TYI-S-33 and the smooth transition between the two enables one to selectively and unhurriedly choose and process vigorous viable organisms. Under the most ideal conditions devised to date, the recovery of viable E. histolytica immediately after thawing is in the order of a few percent. The ability to routinely initiate cultures of E. histolytica with a few hundred amebae has enabled us to obtain vigorous growing cultures in 100% of the samples thawed. Future projected investigations: Improvement in the numbers of amebae recovered can be made by refinement of the cooling rates and procedures for thawing. Attention will be given to optimizing these aspects of the technique. A major effort will be put into exploration of the use of membrane stabilizers, such as spermine and spermidine, in an attempt to minimize the loss of cell membrane integrity which is a common result of freezing injury leading to death of the amebae (Diamond, Claggett, Gillin, Keister and Cunnick).

Role of Iron and Nutritional Immunity in Human Amebic Disease - A clinical and laboratory study aimed at identifying the role of iron in the pathogenesis of human amebic disease which was initiated in 1977 in collaboration with staff members of the Hospital General, Centro Medico Nacional, I.M.S.S., Mexico City has been halted temporarily due to changes in the status of key hospital personnel. However, collating of acquired data in in progress and should be ready for analysis in the near future.

For results of collaborative studies with investigators in other sections within the laboratory see the following annual reports: Project No. Z01 AI 00098-23-LPD, PI E. C. Weinbach, Project No. Z01 AI 00185-01-LPD, PI C. F. T. Mattern.

Publications:

1. Diamond, L. S., Harlow, D. R., and Cunnick, C. C.: A new medium for the axenic cultivation of Entamoeba histolytica and other Entamoeba. Trans. Roy. Soc. Trop. Med. & Hyg. 72: 431-432, 1978.

2. Diamond, L. S., Harlow, D. R., Phillips, B. P., and Keister, D. B.: Entamoeba histolytica, iron and nutritional immunity. Arch. Invest. Med. (MEX) 9 (Suppl. 1): 329-338, 1978.

3. Diamond, L. S., Tanimoto-Weki, M., and Martinez-Palomo, A.: Production of cecal lesions in new born guinea pigs with axenically cultivated Entamoeba histolytica. Arch. Invest. Med. (MEX) 9 (Suppl. 1): 223-228, 1978.

4. Mattern, C. F. T., Keister, D. B., and Diamond, L. S.: Experimental Amebiasis IV: Amebal viruses and the virulence of Entamoeba histolytica. Amer. J. Trop. Med. & Hyg. 28: 653-657, 1979.

5. Gillin, F. D., and Diamond, L. S.: Clonal growth of Entamoeba histolytica and other Entamoeba in agar. J. Protozool. 25: 539-543, 1978.

6. Gillin, F. D., and Diamond, L. S.: Clonal growth of Entamoeba in agar: Some applications of this technique to the study of their cell biology. Arch. Invest. Med. (MEX) 9 (Suppl. 1): 237-246, 1978.

7. Weinbach, E. C., Claggett, C. E., Takeuchi, T., and Diamond, L. S.: Biological oxidations and flavoprotein catalysis in Entamoeba histolytica. Arch. Invest. Med. (MEX) 9 (Suppl. 1): 89-98, 1978.

8. Takeuchi, T., Weinbach, E. C., Gottlieb, M. and Diamond, L. S.: Mechanism of L-serine metabolism in Entamoeba histolytica. Comp. Biochem. Physiol. B. Comp. Biochem. 621B: 281-285, 1979.

9. Gillin G. D., and Diamond, L. S.: Entamoeba histolytica and Entamoeba invadens: Effects of temperature and oxygen tension on axenic growth. Experimental Parasitology (In press).

10. Gillin, F. D. and Diamond, L. S.: Clonal growth of Giardia lamblia trophozoites in a semi-solid Agarose medium. Journal of Parasitology (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00097-21 LPD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Physiological and Cytochemical Pathology of Parasitic Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Teresa I. Mercado Research Physiologist, LPD, NIAID

COOPERATING UNITS (if any) Ms. Alba Colon-Whitt and Dr. Theodore S. Theodore, Laboratory of Streptococcal Diseases, NIAID, Clinical Pathology Dept., NIH

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Physiology and Biochemistry

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
12/12	12/12	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

During the course of studies related to the structure and function of parasite (Trypanosoma cruzi) membranes, contamination of a parasite suspension with a motile, rod-like organism, identified as Pseudomonas fluorescens, occurred which caused lysis of the flagellates. In view of the potential significance of this observation not only in terms of cellular biology and physiology in general, but more specifically, because of its impact on parasite chemotherapy and/or immunology it was deemed advisable to study this interaction more extensively. Our findings are described in this report.

PHS-6040
(Rev. 10-76)

Project Description:

The Tulahuen strain of Trypanosoma cruzi was used. Trypomastigotes were isolated from blood employing column chromatography (Mercado, T. and Katusha, K. 1979. Prep. Biochem., 9: 97-106). Pseudomonas fluorescens was cultured in nutrient broth and resuspended in phosphate buffer-saline (pH 7.4) to an OD of 0.1 at 570 nm. A chemically-defined medium containing sodium glutamate, mannitol, Na^{+}, K^{+}, Ca^{++}, and Mg^{++} was also used. The cultures were incubated for 3, 18, 24, 48, and 72 hours. They were centrifuged at 3,000 rpm (RC3 Sorvall) for 10 min., and the supernatants were filtered through Millipore (0.45 um). The filtrates were checked further for sterility by incubation in blood agar plates and nutrient broth. To examine the trypanosome-bacterium (TB) interaction we mixed 0.15 and 0.30 ml respectively of a parasite-buffer suspension (e.g., 25 X 10^6/ml) with 0.30 ml of the Pseudomonas filtrates. They were examined immediately after mixing, after 10 minutes, and after 12 and 24 hours. The mixtures were maintained at 4° C. In another series the bacteria were sonicated in buffer-saline glucose and this material as well as the supernatant fractions, unfiltered and filtered, were used in the assays. Our assays were based on examination of a drop of the TB mixtures for not longer than a minute because of the possibility that drying of the cover-slipped preparations would interfere with an accurate appraisal of the parasites. They were assessed mainly on the basis of shape and motility. Light and phase microscopy were used. For permanent preparations smears were air dried, fixed in methanol, and stained with Giemsa. In order to ascertain further the occurence of a bacterial exofactor, we concentrated the active fractions following filtration of the supernatant samples. This was done in 3 ways: (1) filtrate was concentrated 10 times, (2) filtrate was dialyzed against distilled water (three, 250 ml changes) and the dialyzate concentrated as described in (1), (3) filtrate dialyzed as in (2) and the dialyzate lyophilized and reconstituted in saline-glucose buffer. The trypomastigotes were isolated immediately after bleeding and the TB mixtures assayed on the same day. All buffers and the DEAE cellulose column were treated with gentamicin at a final concentration of 50 micrograms/ml. The anti-trypanosomal activity of the filtrates was also assessed following incubation at 56° C for 30 minutes, boiling (5 min.), freezing at -20° C and following treatment with trypsin (1 mg/ml) for 30 min. Approximation of the molecular size of the exofactor was made by filtration employing filters of different porosities. An important consideration from these observations was whether or not the modified parasites would induce infection and/or be protective against a challenge with parasitized blood. Four animals were inoculated intraperitoneally with 8 X 10^6 trypomastigotes of which only 5% were typically motile, lanceolate organisms. In another experiment, 2 animals were inoculated with 0.4 and 0.5 ml respectively of a suspension of sonicated cells. The animals were challenged after one month with freshly-drawn blood containing 1 and 2 X 10^6 trypomastigotes.

In the presence of P. fluorescens, trypomastigotes of T. cruzi are considerably immobilized. Usually one bacterium approaches a single flagellate and with persistent to and fro movements directed to the posterior end

(kinetoplast-flagellar site) it quickly subdues the flagellate. Giemsa-stained preparations revealed a rounding-up of the parasite following the initial encounter; the flagellum appeared wrapped around the cell body and as the incubation time increased disintegration of this structure and of the nucleus occurred. The cell bodies later became considerably reduced in size and assumed bizarre triangular and rounded shapes. Filtrates of supernatant fractions of the centrifuged bacterial cultures caused immobilization and accompanying cellular disintegration within 12 hours; however, an almost instantaneous paralysis followed by lysis occurred when the filtrates were concentrated. Cell membranes were scattered about and some parasites though still intact, were noticeably swollen and their protoplasm became transparent with an apparent loss of organelle boundaries. Cultures grown in nutrient broth exhibited an optimal immobilizing effect after incubation for 48 hours; the immobilization response however, was also observed following incubation for 18 and 24 hours. In contrast to nutrient broth, media 36, though a good medium for the culture of P. fluorescens, was poor for the production of the lytic factor. Only in media 36 inoculated from broth cultures was the lytic factor produced in sufficient amounts to elicit the immobilization response and lysis of the parasites. Preliminary experiments on the characterization of the lytic factor disclosed that it is resistant to heat, freezing, is not inhibited by trypsin, and has a molecular weight lower than 10,000 daltons. We did not observe typical Tulahuen strain parasitemias in animals inoculated with the T. cruzi bizarre forms. Three of 4 animals became infected, but the highest parasitemia (observed after 10 days) was 29 per 30 microscopic fields examined. The fourth animal did not become infected. No alteration of the chronic infection was elicited following a challenge with 1 X 10^6 flagellates, based on the examination of a drop of blood, the animals had become negative after 33 days. Similarly, animals inoculated with a suspension of P. fluorescens sonicated cells did not develop lethal infections following a challenge, after one month, with 1 and 2 X 10^6 trypomastigotes respectively; very mild parasitemias were observed (e.g., 6 parasites per 30 microscopic fields).

Our studies on the characterization of the lytic factor are being continued. However, based on the microscopic observations of the flagellate-bacterium interaction, in the apparent absence of cell lysis, and the significant lysis produced by concentrated dialyzed filtrates, we believe the active factor is secreted extracellularly and the observed effects are not a consequence of bacterial cell lysis. This assumption is substantiated further by the fact that supernatant fractions of sonicated cells elicited essentially a rounding-up of the parasite with the production of bizarre forms and not a lytic effect. The extracellular factor have so far disclosed certain properties which are comparable to some P. fluorescens exoenzymes reported by other investigators.

Although our animal inoculation results are only preliminary, they are interesting particularly in view of a report (Bomford, R. and McHardy, N. 1979. Parasitology 78: 77-87) in which they described an enhancement of

the protective effect of a T. cruzi epimastigote vaccine in the presence of the bacterial species, Corynebacterium parvum. The apparent protective effect resulting in our study of the P. fluorescens-T. cruzi interaction is currently being examined more extensively. The influence of other bacterial species such as Escherichia coli and P. aeruginosa as well as species of African trypanosomes which multiply in the blood stream, not in tissues, will also be studied. Changes produced on the parasite membrane will also be studied biochemically and cytochemically.

The present study though not projected as part of the main program related to the physiological and cytochemical pathology of parasitic disease has served to emphasize the importance of research on parasite interactions and their potential significance leading to a chemotherapeutic or immunologic control of parasitic infections.

Publications:

1. Mercado, T. I. and Katusha, K.: 1979. Isolation of Trypansoma cruzi from the blood of infected mice by column chromatography. Prep. Biochem., 9:97-106.

2. Mercado, T. I. and Garbus, J.: Creatine phosphokinase isoenzymes and Trypanosoma cruzi infections. Comp. Biochem. Physiol. (In press).

3. Mercado, T. I.: 1979. Observations on lactate dehydrogenase isozymes in the plasma of mice infected with Trypanosoma cruzi. Isozyme Bull. 12:39.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00098-23

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Biochemical mechanisms of energy metabolism in mammalian and parasitic organisms.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	D. M. Dwyer	Research Microbiologist, LPD, NIAID

COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Physiology and Biochemistry

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
36/12	22/12	14/12

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The study of aerobic energy metabolism in parasites and mammals was continued with efforts devoted primarily to elucidating the sequence of electron transfer in the respiratory chain of Entamoeba histolytica. Unique biochemical properties of the amebae are their absence of heme proteins, large content of non-heme iron and acid-labile sulfide, minute amounts of quinones, and absence of covalently bound flavins. Hydrogen peroxide is formed only when artificial electron carriers mediate electron transfer from substrates to molecular oxygen, indicating that iron-sulfur centers and not flavin is the final electron carrier. Biochemical studies of Giardia lamblia were initiated this year. Respiratory metabolism of G. lamblia resembles that of E. histolytica by its lack of a functional tricarboxylic acid cycle and cytochromes; mediating electron transfer by flavoproteins and non-heme iron proteins. Only a small amount of acid-labile sulfide was detected in G. lamblia. Differences in the locus of the respiratory enzymes and substrate specificities are clearly evident in the aerobic metabolism of these two enteric protozoa. Another study initiaed this year is the examination of respiratory metabolism

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of kinetoplasts from Leishmania donovani. Respiration was supported by Kreb's cycle substrates, particularly succinate, and the presence of functional NADH oxidoreductose was demonstrated. Mammalian studies centered on the assessment of whether protein synthesis or respiration was first affected during cellular heme depletion in cultured murine erythroleukemia cells.

Project Description:

The object of this project is to conduct fundamental studies on the mechanisms of aerobic energy metabolism in mammalian and parasitic organisms. Currently these studies center primarily on identification and characterization of components of the respiratory chains of pathogenic protozoa, and elucidation of the sequence of electron flow to molecular oxygen.

Tissues and cells are disrupted mechanically and subcellular fractions are isolated by differential and gradient centrifugations. Cellular and subcellular constituents, enzyme activities, and metabolic pathways are determined, characterized and elucidated by enzymatic, chemical, radiochemical and physical methods. Changes in oxygen concentration are determined polarographically with the Clark electrode. Electron probe microanalysis is used to localize elements in cestode calcareous corpuscles.

Parasite studies: Continued investigation of the respiratory chain in Entamoeba histolytica, strain HK-9, cultivated axenically in Diamond's liquid medium, was focused primarily on elucidating the sequence of electron flow to molecular oxygen. We now have identified the following electron carriers in E. histolytica: nicotinamide adenine nucleotides, flavoproteins, minute amounts of quinones, and iron-sulfur centers. Combined use of inhibitors and spectrophometric analyses have shown that the sequence of electron flow is from reduced nicotinamide adenine nucleotides (NAD(P)H) to flavins to iron-sulfur centers (Weinbach, Takeuchi and Claggett). Electron paramagnetic resonance (EPR) studies and direct chemical analyses have revealed the presence of multiple iron-sulfur centers in E. histolytica. These centers are one-electron tranfer proteins which lead to the production of H_2O. Flavins, in contrast, are two-electron transfer carriers and lead to the production of H_2O_2. We did not detect H_2O_2 as an end product of aerobic metabolism in E. histolytica; therefore, we conclude that the terminal acceptor in this parasite is an iron-sulfur center, rather than flavin as claimed by others (Weinbach and Claggett). Our studies of the enteric protozoa were extended to another human pathogen, Giardia lamblia. In collaboration with Diamond and Keister, the axenically-cultivated trophozoites were found to have active endogenous respiration (32 nanomoles O_2/min/mg protein) and a high affinity for oxygen. Substrate specificity and inhibitor sensitivity indicated that this eukaryote, which, like E. histolytica, also lacks mitochondria, is devoid of tricarboxylic acid cycle enzymes and cytochromes. Heme iron could not be detected by chemical or EPR analysis. G. lamblia exhibits unusual

EPR signals and has a low content of acid-labile sulfide. This parasite may contain non-heme proteins of the rubredoxin-type and only small amounts of the ferredoxin-type (Weinbach and Kon). Alcohol dehydrogenase, which is exceedingly active in E. histolytica, is absent in G. lamblia. Sonicated trophozoites were equally effective in oxidizing NADH or NADPH, demonstrating the presence of a functional NAD(P)H oxidoreductase. Separation of the particulate and soluble fraction of G. lamblia disclosed that the particulate fraction contained most of the reductase activity and required no exogenous carrier to mediate electron transfer to molecular oxygen. Spectrofluorometric analysis showed that virtually all of the flavin was acid-extractable and not covalently bound (Claggett). Flavo-antagonists inhibited the respiration of intact trophozoites, and NAD(P)H oxidoreductase activity. Another study that initiated this year resulted from a "spin-off" from Dr. Dwyer's work on the cell membranes of Leishmania donovani. During the course of cell fraction of this parasite, suspension of clean but fragmented kinetoplasts were obtained. Our preliminary studies of these preparations reveals their capacity to oxidize a number of tricarboxylic acid cycle substrates. Oxidation of succinate was particularly vigorous (V_{max} = 200 nanoatoms/min), and is extremely sensitive to malonate. Unlike its mammalian counterpart, the malonate inhibition was not overcome by addition of excess substrate. Electron transfer to molecular oxygen, unlike that of the enteric amebae, is through the cytochromes. Respiratory control, indicative of oxidative phosphorylation, is absent in these fragmented kinetoplasts (Weinbach and Dwyer).

Mammalian studies: The previous collaboration with Dr. Tschudy, NCI, on the relation of heme biosynthesis and biological oxidations was resumed with a study of respiratory phenomena in cultured murine erythroleukemia (MEL) cells. The synthesis of hemoglobin in these cells is strongly inhibited by succinylacetone. Examination of MEL cells' respiratory capacity showed an active endogenous respiration and the presence of an NADH: (quinone acceptor) oxidoreductase. There was little difference in these activities in cells treated with succinylacetone. Our preliminary data show that although cell growth and hemoglobin synthesis was markedly diminished by the succinylacetone, respiration was unaffected (Weinbach, Ebert and Tschudy).

This project, which attempts to elucidate vital biochemical mechanisms common to all aerobic cells, has obvious implications for biomedical research in general (illustrated by the numerous collaborative projects), and provides a rational basis for understanding bioenergetic mechanisms associated with parasites and parastism. The parasite studies will be continued with emphasis on identifying the ultimate electron carrier in E. histolytica and G. lamblia that replaces cytochromes in higher eukaryotic organisms. We hope to purify the amebal NAD(P)H (quinone acceptor) oxidoreductase by affinity chromatography utilizing a new technique of menadone (the acceptor) bound to sephorose. This technique

also should enable us to separate the oxidoreductase from other amebal enzymes, particularly glutathione reductase. We have begun preliminary experiments with this enzyme which appears to be of physiological importance in both E. histolytica and G. lamblia, based on the studies of Dr. F. Gillin, LPD. (see her report). A study of iron-sulfur electron transfer proteins in G. lamblia appears to be particularly promising in view of the marked differences from those of E. histolytica. The rewarding preliminary experiments with kinetoplasts from L. donovani will be expanded with the objective of elucidating the respiratory mechanisms of this organelle. The mammalian work on the effects of succinylacetone on cultured murine erythroleukemia cells will be expanded to other aspects of hemoglobin biosynthesis. A report of our initial findings is in preparation. Because of shortage of manpower the expected collaboration on the study of energy metabolism in human blood platelets was not resummed this year. We hope that this can be done during the ensuing year. Unfortunately, Dr. Takeuchi had to return to Japan earlier than expected during the past year and he could not finish his work on isopropanol reductase in E. histolytica. We now have done this and a paper is in preparation. The microprobe analysis of metal distribution in cestode calcareous corpuscles was done in Dr. Barnard's laboratory in Sweden and a few samples were analyzed by the Tousimis Research Corporation in Bethesda. Although difficulty was experienced by both groups in sectioning the samples, differences were observed in the distribution of metals between the matrix and outer shell of the corpuscles. The untimely death of Dr. T. von Brand temporarily suspended this work. The data must be collated and analyzed to ascertain if sufficient information is available for publication.

Publications:

1. Weinbach, E. C., Claggett, C. E., Takeuchi, T. and Diamond, L. S.: Biological oxidations and flavoprotein catalysis in Entamoeba histolytica. Arch. Invest. Med. 9: 89-98 (1978).

2. Takeuchi, T., Weinbach, E. C., Gottlieb, M. and Diamond, L. S.: Mechanism of L-serine metabolism in Entamoeba histolytica. Comp. Biochem. Physiol. B. Biochem. 62B: 281-285 (1979).

3. Weinbach, E. C. and Bueding, E.: Theodor von Brand: A Tribute. J. Parasitol. 65: 182-184 (1979).

4. Pazoles, C.J., Claggett, C.E., Creutz, C.E., Pollard, H.B, and Weinbach, E.C.: Identification and subcellular localization of catalase activity in bovine adrenal medulla and cortex. Arch. Biochem. Biophys. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00099-09 LPD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Biophysical Parasitology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	G. A. Schmunis	Visiting Scientist, UFRJ, Brazil

COOPERATING UNITS (if any)
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Laboratory of Parasitic Diseases

SECTION
Physiology and Biochemistry

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NIAID, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A multiparametric approach utilized to study the interaction of Trypanosoma cruzi with vertebrate cells indicates that the infection of vertebrate cells by T. cruzi is an active process. Cytochalasin does not inhibit the infection of non-phagocytic vertebrate cells by trypomastigotes. Chronic chagasic serum enhances the ability of trypomastigotes to penetrate vertebrate cells. The phenomenon is not specific to parasite strain, host cell type or serum source. Total immunoglobins are capable of enhancing penetration. However, purified specific immunoglobulin fractions are inactive. Activity is restored to the IgG fraction with an as yet undefined factor present in normal human serum. The first round of DNA synthesis by a population of intracellular amastigotes is synchronous. Synchrony decays during subsequent parasite divisions. The confirmation of a lag period prior to the initial round of DNA synthesis indicates that trypomastigotes are in an extended G_1 [G_o] phase prior to infection of a vertebrate cell. A group of vertebrate cell X T. cruzi hybrids have been produced utilizing P3-X63Ag8 vertebrate cells and T. cruzi epimastigotes. The vertebrate cell hybrids express T. cruzi antigen as demonstrated by IFA. Antigen expression has been stable for 14 weeks.

Project Description:

A multiparametric approach is being utilized to study the interaction of Trypanosoma cruzi with vertebrate cells. Five major topics are presently being studied: 1. Elucidation of the mechanism of entry of a trypomastigote into a non-phagocytic cell; 2. The effect of immune serum on the ability of a trypomastigote to attach to and, subsequently, penetrate a vertebrate cell; 3. The patterns of macromolecular synthesis and utilization of precursors by host cells and parasites during the intracellular phase of the cycle; 4. Elucidation of the mechanism responsible for the transformation of epimastigotes to trypomastigotes and; 5. The production of T. cruzi X vertebrate cell hybrids that express T. cruzi antigens.

1. Mechanism of entry of trypomastigotes into non-phagocytic vertebrate cells

It has been postulated that phagocytosis is the sole mechanism of infection of vertebrate cells by trypomastigotes. Data to support the postulate were collected from studies of macrophages which are normally phagocytic. The entry of trypomastigotes into non-phagocytic cells is postulated as being due to the induction of vertebrate cell phagocytosis by the parasite. This concept is at variance with data collected in this laboratory. For example, as reported previously, protease inhibitors inhibit the penetration of fibroblasts by trypomastigotes to the same extent as protease + inhibitor. Consequently, it would appear that inactivation of labile enzymes present on host cell or parasite rather than removal of receptors is a better interpretation of the data. In addition, it has been reported that Cytochalasin inhibits the penetration of macrophages by trypomastigotes. A recent study in this laboratory has demonstrated that Cytochalasin does not inhibit the penetration of fibroblasts by trypomastigotes. Furthermore, when tested together as a mixed system, the ability of trypomastigotes to penetrate fibroblasts is many-fold greater than macrophages. Under identical conditions, macrophages rapidly phagocytosed sheep red blood cells whereas the fibroblasts did not. Although still indirect, these data lend further support to the hypothesis that trypomastigotes are capable of actively penetrating vertebrate cells.

2. Influence of immune serum on the ability of T. cruzi trypomastigotes to penetrate vertebrate cells

T. cruzi antiserum has a lytic, agglutinogenic and opsonizing effect upon trypomastigotes and may induce redistribution and loss of surface antigens. The fact that immune serum may partially protect against lethal infection of mice when passively transferred could indicate that T. cruzi antibodies may have an inhibitory effect upon infection of vertebrate cells. To test this possibility, trypomastigotes were incubated with human fibroblasts (WI-38) in the presence of normal or chagasic human serum and the subsequent infection quantified. It was observed that: 1. human chagasic sera increase the attachment and penetration of blood trypomastigotes from 3 different strains of parasite. 2. Dialysis of chronic chagasic serum slightly decreases

attachment and penetration compared to non-dialyzed serum. However, penetration is still greater than observed with normal serum. 3. the factor(s) responsible for increasing attachment and penetration are in the $\geq$ 50,000 MW fraction of the serum. 4. The total immunoglobulins have essentially the same activity as whole serum. However, the IgM and IgG fractions are inactive. Activity was restored when the IgG (but not IgM) fraction was mixed with fresh human normal serum. 5. Absorption of chagasic serum was Protein-A or epimastigotes decreased attachment and penetration. Absorption with WI-38 cells or sheep red blood cells had no effect. 6. Fixed trypomastigotes incubated with chagasic serum do not attach to WI-38 cells. Serum from chronically infected rabbit, mouse or monkey increases the penetration of WI-38 cells by homologous or heterologous trypomastigotes. 7. Human chagasic and chronic rabbit sera increase the penetration of BESM cells by trypomastigotes.

3. Studies of the pattern of DNA synthesis of host cells and parasites during the intracellular phase of the cycle

DNA synthesis of intracellular T. cruzi amastigotes following the infection of BESM cells, was studied by autoradiography. After penetration, there was a pre-replicative lag period ($\sim$ 12 hr) followed by a synchronous round of DNA synthesis which was found to be independent of parasite number/BESM cell and the host cell DNA synthesis cycle. Parasite reproduction occurred, for the first time, at $\sim$ 21 hr post infection. It was concluded that T. cruzi trypomastogotes are in the G_0/G_1 phase of their cell division cycle and, after penetration, parasite reproduction occurs independent of events controlling the host cell DNA synthesis-growth cycle. The early synchronous growth of intracellular amastigotes should facilitate further studies on the biochemical events controlling trypomastigote-to-amastigote reproduction. A further application is envisaged for studies involved with the mode of action of drugs with trypanocidal activity.

4. Elucidation of the mechanism responsible for the transformation of epimastigotes to trypomastigotes.

The transformation of T. cruzi from one morphologic form to another occurs naturally at several points in the life history of the parasite. One of these transformations, epimastigote to trypomastigote, was chosen for study in an attempt to elucidate the conditions required for transformation to occur. Thus far, numerous substances known to induce transformations in vertebrate cells have been tried. These include 0-20 mM thymidine and 0-20 mM dibutryl-cyclic AMP, 0-5 mM adenosine and 0-5 mM monobutryl-cyclic GMP which have no effect on growth or morphology. Adenosine (10-20 mM), 10 mM monobutryl-cyclic GMP and 5 mM theophylline inhibit growth of epimastigotes but no transformation to trypomastigotes occurs. Although all of these data represent negative experiments with respect to transformation, they point out clearly that the biochemistry of transformation of these parasites is obviously different from that found in vertebrate cells.

5. The production of vertebrate cell hybrids that express T. cruzi antigens

The production of vertebrate cell X T. cruzi hybrids initiated last year has been continued. The technique for selection of hybrid clones expressing T. cruzi antigen is the only major problem remaining that requires development to make the entire system practical. This problem can probably be overcome using affinity columns or a cell sorter. However, three clones have been established utilizing a P3-X63Ag8 vertebrate cell line and epimastigotes as starting material. The P3-X63Ag8 cell line is HAT sensitive which allows for selection of hybrids along more classical lines. Thus far, all three hybrid clones express T. cruzi antigen as demonstrated by IFA.

Aside from the obvious usefulness of this system to basic cell biological questions, there are several major Chagas' disease-related problems that can now be studied. The parasite antigens can be isolated, characterized and used as specific immunogens. It is not yet known if all hybrid cell lines express the same antigens. If not, the cell hybrids could provide a model for the study of possible immunopathologic lesions resulting from T. cruzi infection.

Significance and Proposed Course of Program:

(1) The study of host-parasite interactions at the cellular and sub-cellular level is a topic of critical importance. The outcome of this interaction directly influences the physiologic state and survival potential of both the host and the parasite.

(2) The methodology and research philosophy being developed are directly applicable to other areas of biomedical research. For example, the controlled-environment cluture system developed with this program is now in routine use in laboratories with such diverse interests as oncology, immunology, neuro-physiology and morphogenesis. The video systems developed with this program are being used for analysis of macroscopic as well as microscopic images in basic research and diagnostic clinical-medical fields.

Research will continue in an attempt to further our understanding of the interaction of T. cruzi with vertebrate cells in vitro and relate this information to the course of Chagas' disease in nature. Basic information obtained with the controlled-environment culture system and video systems will be utilized in more complex in vitro model systems.

The basic methodology and research philosophy developed with this program will be used for the analysis of other host-parasite interactions.

Publications:

1. Dvorak, J. A. and Howe, C. L: Toxoplasma gondii-vertebrate cell interactions: II. The intracellular reproductive phase. J. Protozool. 26: 114-117, 1979.

2. Weller, P. F., Dvorak, J. A. and Whitehouse, W. C.: Human eosinophil stimulation promoter lymphokine: Production by antigen stimulated lymphocytes and assay with a new electro-optical technique. Cellular Immunol. 40: 91-102, 1978.

3. Dvorak, J. A. and Schmunis, G. A.: The influence of acute and chronic human chagasic sera on the ability of Trypanosoma cruzi to attach to and penetrate human diploid fibroblasts (abs, 4th Int. Congress of Parasitol., 1978, Vol. E, pp 26-27.

4. Dvorak, J. A.: Letter to the Editor. J. Parasitol. 26: 158, 1979.

5. Dvorak, J. A.: Trends in the use of in vitro cell cultures for Trypanosoma cruzi research. in In Vitro Cultivation of Pathogens of Tropical Diseases. (In press).

6. Dvorak, J. A.: In vitro studies of the interaction of Trypansoma cruzi with vertebrate cells. International Congress on Chagas Disease, Rio de Janeiro. (In press).

7. Crane, J. St. J. and Dvorak, J. A.: Studies on DNA synthesis during the intracellular cycle of Trypanosoma cruzi: Host-parasite inter-relationhsip. International Congress on Chagas Disease, Rio de Janeiro. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00102-05 LPD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Pathogenesis of Disease Caused by Infection with Intracellular Parasites

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	F.A. Neva	Chief, Laboratory of Parasitic Diseases, NIAID
Others:	Renato Gusmao	Visiting Fellow, LPD,NIAID
	Albert Gam	Biological Laboratory Technician (Micro.), LPD,NIAID

COOPERATING UNITS (if any) Professors Joffre Rezende and Anis Rassi, University of Goias, Goiania, Brazil. Professor Aluizio Prata, University of Brasilia, Brasilia, Brazil. Dr. Frances Ward, Duke University Medical Center, Durham, N.C. Dr. Euripides Ferreria, University of Parana, Curtiba, Brazil.

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Cell Biology and Immunology Section

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
27/12	16/12	11/12

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The association of certain HLA-tissue types with chronic Chagas' disease noted previously could not be confirmed in a much expanded study. B-cell typing remains to be checked and basic immunogenetic information concerning the Brazilian population has been a useful by-product of the study.

The plaquing technique for intracellular phase T. cruzi shows promise as a tool for clonal selection and analysis of isolates of the parasite. Other applications of cell culture for characterization of strains of T. cruzi include temperature tolerance and growth curves.

Use of the foot-pad site for inoculation of BALB/c mice and subsequent measurements of the lesion has proved to be a useful model for experimental work with strains of cutaneous leishmaniasis.

PHS-6040
(Rev. 10-76)

Project Description:

The variable geographic distribution of the late sequelae that constitute chronic Chagas' disease suggest that genetic factors may be involved in development of the chronic disease. Therefore, it was considered important to thoroughly explore possible association of HLA-tissue types with chronic Chagas' disease. A preliminary study last year disclosed correlations between certain forms of chronic Chagas' disease and presence as well as absence of certain HLA types. However, the study groups were relatively small (12 to 18 cases) so it was necessary to expand the numbers of patients studied, and family members were also included. Again such a study required rather complicated logistics and collaboration of other groups for patient selection and the HLA-typing at A, B and C loci. A controlled freezing apparatus was also transported to Brazil so lymphocytes could be harvested from patients, frozen and brought back to the U.S. for D-locus typing. After review of clinical and laboratory findings between 36 and 53 patients were available for analysis. In this expanded study the apparent correlations of certain HLA types with chronic Chagas' disease in last year's study could not be confirmed. B-cell typing (DRw) on the frozen cells has not yet been performed, but will be done. Quite apart from possible HLA relationships with a parasitic infection, this study has disclosed some findings of intrinsic interest to immunogenetics. For example, the genetic diversity of this Brazilian population is unique and several new HLA specificities will probably emerge from the family studies. The fact that the HLA background and status of Chagas' disease has been established in a population that can be followed may make it worthwhile to monitor the study groups for the next several years for evolution of their disease state. (Neva, Gusmao, Gam and collaborators).

The assessment of different isolates of Trypanosoma cruzi, especially activity of the intracellular phase of the organism, using cell cultures as a host system has continued. Technical features of the intracellular plaquing procedure have been standardized. The ability of isolates or strains to form plaques is correlated with minimal infective dose for cell cultures as titrated in serial dilutions, and with growth curves over 3 cycles of parasite development. The minimal infective dose does not necessarily correlate with the ability of a strain to produce plaques. There is evidence that the plaquing technique can be used for cloning isolates of T. cruzi because successive plaque isolation appears to enhance a property of the isolate, such as ability to produce plaques at an elevated temperature of 38°C. Although most isolates of T. cruzi have been found to grow optimally at 33°C, a recent isolate from a Brazilian patient with acute Chagas' disease was found to grow better at 38°C. All observations lend support to the concept that assessment of biologic activity of T. cruzi by cell culture techniques is a valid approach and that the plaquing procedure will be useful for clonal selection and analysis of populations of T. cruzi. (Neva and Gam).

Further work has been done on the foot-pad infection in the genetic in-bred BALB/c mouse with leishmania causing cutaneous leishmaniasis. Use

of this inoculation site and subsequent measurement of width and thickness of lesions at weekly intervals for quantitative assessment of lesion size has proved to be a useful model for experimental work. A previous experiment with resistant C57 Bl and DBA mice, their F1 progency with BALB/c and back-cross with BALB/c gave results suggesting a single gene inheritance pattern for susceptibility. A similar experiment repeated with DBA and BALB/c mice was still equivocal although similar to what would be expected of a single gene effect. A distinctly reduced foot-pad size in several experiments with a standard strain of L. tropica was initially thought to represent loss of virulence of the strain. However, the decreased response was found due to use of a smaller inoculum, and this dose-response relationship was clearly demonstrated with another strain of L. tropica as well. Reproducibility of the foot-pad response was shown with strains of L. tropica after storage in liquid nitrogen. Although factors such as inoculum size can influence response with this system, the foot-pad size seems basically to reflect intrinsic virulence of the strain of leishmania. (Neva).

Publications:

1. Neva, F.A., Wyler, D.J., and Nash, T.E.: Cutaneous leishmaniasis - A case with persistent organisms after treatment in presence of normal immune response. Am. J. Trop. Med. Hyg. 28: 467-471, 1979.

2. Bjorvatn, B., and Neva, F.A.: A model in mice for experimental leishmaniasis with a West African strain of Leishmania tropica. Am. J. Trop. Med. Hyg. 28: 472-479, 1979.

3. Bjorvatn, B., and Neva, F.A.: Experimental therapy of mice infected with Leishmania tropica. Am. J. Trop. Med. Hyg. 28: 480-485, 1979.

4. Ottesen, E.A., Neva, F.A., Paranjape, R.A., Tripathy, S.P., Thiruvengadam, K.V., and Beaven, M.A.: Specific allergic sensitisation to filarial antigens in tropical eosinophilia syndrome. Lancet: 1158-1161, June 2, 1979.

5. Ottesen, E.A., Hiatt, R.A., Cheever, A.W., Sotomayor, Z.R. and Neva, F.A.: The acquisition and loss of antigen-specific cellular immune responsiveness in acute and chronic schistosomiasis in man. Clin. exp. Immunol. 33: 38-47, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AI 00103-12 LPD

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Immunological Studies on Toxoplasmosis and Other Parasitic Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. N. Lunde	Research Zoologist
Other:	A. W. Cheever	Assistant Chief, LPD, NIAID
	E. A. Ottesen	Senior Investigator, LPD, NIAID
	T. E. Nash	Senior Investigator, LPD, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Parasitic Diseases

SECTION

Host-Parasite Relations

INSTITUTE AND LOCATION

NIAID, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
18/12	13/12	5/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cercarial/adult (c/a) antibody ratios from acutely infected patients were higher than from individuals with chronic schistosomiasis. Specific IgM titers to soluble egg antigen (SEA) were found in all patients with acute or early schistosomiasis but from only a few patients with chronic schistosomiasis. IgM antibody in the chronic patients correlated with the presence of circulating immune complexes. Specific IgE antibody was found in most of the sera from acute patients and in only a few of the ones with chronic schistosomiasis. SEA and toxoplasma antigen added to serum have been detected by ELISA at levels of 10 and 100 ng/ml respectively.

PHS-6040
(Rev. 10-76)

Project Description:

Emphasis has been focused on improving the enzyme-linked immunosorbent assay (ELISA) for serodiagnosis and study of host parasite immune responses in parasitic diseases, especially schistosomiasis and toxoplasmosis. The ELISA is used to measure immunoglobulin class specific antibodies to antigens prepared from different stages of the life cycle of Schistosoma mansoni and also to quantitate antigens from these life cycle stages. The following findings relative to schistosomiasis were established:

1. Sera from patients with acute schistosomiasis were found to have higher ELISA titers with cercarial antigen than with adult antigen. In contrast sera from patients with chronic schistosomiasis were found to have higher titers with adult worm antigen. This difference could be quantified and expressed by determining the ratio of cercaria/adult (c/a) antigen OD_{490nm} extinction values at a 1:16 dilution of serum.

2. Acute patients, those with high c/a ratios had schistosome specific IgM antibody but only 2 out of 11 patients with chronic schistosomiasis had positive IgM titers. Interestingly these two patients were also the only ones of the group found to have circulating immune complexes. (Lawley et al., to be published). These observations suggest a possible link between the determinants which lead to chronic IgM production and those responsible for the persistence of circulating immune complexes.

3. IgE antibody was demonstrated in 11 of 13 acutely infected patients with titers ranging from 1:8 to 1:64 while sera from all chronically infected persons except one were negative for IgE antibodies in the ELISA. Schistosome IgE was demonstrated almost exclusively with egg antigen as cercarial and adult worm antigens elicited only negative or weakly positive responses. One interpretation of these observations is that the schistosome egg antigens play a predominant role in eliciting hypersensitivity responses in the infected host.

4. Fab' fragments of IgG from a monkey infected with S. mansoni have been labeled with horseradish peroxidase and used in an ELISA procedure to detect antigen. Using this procedure soluble egg antigen (SEA) can be detected as low as 10 ng/ml when added to 10% serum in PBS. This is being done as a prelude to hopefully being able to detect circulating antigen and antigen moities of immune complexes.

The diagnosis of toxoplasmosis is usually based on demonstration of antibodies by different serological methods. However, for confirmation of active toxoplasmosis it would be helpful if a method could be obtained for the detection of circulating antigen. Using Fab' fragments of IgG from a rabbit infected with toxoplasms which have been conjugated with peroxidase toxoplasma antigen has been detected at the 100 ng/ml level when added to 10% serum.

Proposed Course:

1. As sera becomes available from patients post treatment for schistosomiasis we plan to use ELISA to study immune response after treatment. If changes can be found in levels of specific immunoglobulins with antigens from different stages of the life cycle then we have a handle for evaluating chemotherapy.

2. Methods currently available for the detection of circulating antigen in schistosomiasis have used radiolabeled antibody or counter current immunoelectrophoresis. Perhaps ELISA methodology offers a means of detecting such antigen in human subjects. In the presence of circulating antibody these antigens can form complexes which, indeed, have been demonstrated in schistosomiasis. If ELISA proves successful in measuring circulating immune complexes, then complexes of other parasitic diseases will be studied. Along these lines we hope to eventually separate and identify the antigen and antibody moities of these complexes since they can be considered a pathogenic mechanism in parasitic diseases.

3. Improvements in the ELISA techniques to detect circulating antigen in tomoplasmosis will be continued.

Significance to Biomedical Research:

Application of ELISA for multipurpose seroepidemiological and clinical studies is being contemplated in areas endemic for malaria, trypanosomiasis and schistosomiasis by the World Health Organization. We have shown how ELISA can be a useful tool in distinguishing acute and chronic schistosomiasis thus making it useful for seroepidemiological and clinical studies. Further investigations employing ELISA to detect circulating antigens and circulating immune complexes should be valuable from a diagnostic standpoint and also as a means of identifying the role of complexes in the disease process.

Publications:

1. Lunde, M. N., Ottesen, E. A. and Cheever, A. W.: Serological differences between acute and chronic Schistosomiasis mansoni using enzyme-linked immunosorbent assay (ELISA). Am. J. Trop. Med. Hyg. 28: 87-91, 1979.

2. Lunde, M. N. and Ottesen, E. A.: Enzyme-linked immunosorbent assay (ELISA) for detecting IgM and IgE antibodies in human schistosomiasis. Am. J. Trop. Med. Hyg. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00108-08 LPD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Studies on the Biology and Immunogenicity of Malarial Sporozoites.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: R. W. Gwadz — Research Entomologist, LPD, NIAID

Other: R. S. Nussenzweig — Professor and Head, Division of Parasitology, NYU School of Medicine

COOPERATING UNITS (if any)
New York University, School of Medicine

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Malaria Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
9/12	3/12	6/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study the immune mechanisms involved in sporozoite-induced infections, and to develop protocols for immunization with sporozoite antigens. Studies underway are concerned with development of methods for immunizing rhesus monkeys with Plasmodium knowlesi sporozoites, and characterization of the structure, function and immunogenicity of surface determinants of sporozoites.

PHS-6040
(Rev. 10-76)

Sporozoite immunization

Rhesus monkeys have been immunized by intravenous injection of sporozoites of the H strain (Malaysian) of P. knowlesi. Animals resistant to challenge by bite of P. knowlesi infected mosquitoes showed consistently high levels of anti-sporozoites antibodies as determined by circum-sporozoite precipitin (CSP) reactions and immunofluorescense (IFA). In addition, serum from protected monkeys inactivate sporozoites after a 1 hour incubation in a test for sporozoite neutralization activity (SNA).

Monkeys that received similar quantities of sporozoite antigen but failed to develop antibodies as determined by CSP, IFA or SNA tests were not resistant to challenge by bite of infected mosquitoes. We can demonstrate a positive correlation between these serological tests and the presence of protective immunity against sporozoites.

Passive transfer of serum from immune donor monkeys to non-immune recepients confers complete protection in the recepient against sporozoite challenge, demonstrating the critical role of antibody in the anti-sporozoite reaction.

Characterization of sporozoite surface antigens

P. knowlesi sporozoites were radiolabelled by lactoperoxidase mediated iodination and disrupted by French pressure cell. SDS--polyacrylamide gel electrophoresis, followed by autoradiography revealed the presence of a small number of labelled proteins in the extract. Immunoprecipitation with specific antisera to P. knowlesi detected primarily one of these membrane components, with molecular weight of approximately 100,000 daltons.

Proposed course:

The following investigations will be pursued in the coming year with the eventual goal of immunization against sporozoites of the human malarias.

1) improvement of the P. knowlesi/monkey model with respect to immunization schemes.

2) characterization of the sporozoite surface antigens most responsible for immunity. The techniques of metabolic labelling and hybridoma production of specific antibodies are being used to facilitate this characterization.

3) non-response of some monkeys to sporozoite antigens and resulting non-protection has stimulated an examination of histocompatability types in rhesus monkeys.

Significance:

A malaria vaccine effective against mosquito-injected sporozoites would be of great importance to human populations at risk in much of the tropical

world. The ready application of this type of immunization has met with a number of practical difficulties. The development of a Simian model for studies of the mechanisms and methodology for inducing anti-sporozoite immunity would be very useful for the solution of some of these problems. In addition, characterization of the functional sporozoite antigens could lead to synthesis and elimination of unreasonable dependence on mosquito-produced sporozoite antigens for immunization.

Publications:

1. Gwadz, R. W., Cochrane, A. H., Nussenzweig, V. and Nussenzweig, R. S.: Preliminary studies on vaccination of rhesus monkeys with irradiated sporozoites of Plasmodium knowlesi and characterization of surface antigens of these parasites. Bull. WHO (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00109-07 LPD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Cellular Immunology of Malaria and Other Parasitic Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. J. Wyler	Senior Investigator, LPD, NIAID
Other:	C. N. Oster	Senior Staff Fellow, LPD, NIAID
	T. C. Quinn	Research Associate, LPD, NIAID
	J. D. Berman	Clinical Associate, LCI, NIAID
	L. M. Wahl	Senior Staff Fellow, LMI, NIDR
	S. M. Wahl	Research Microbiologist, LMI, NIDR
	A. W. Cheever	Assistant Chief, LPD, NIAID
	D. M. Dwyer	Research Microbiologist, LPD, NIAID

COOPERATING UNITS (if any) Laboratory of Microbiology and Immunology, NIDR, Dept. of Anatomy and Cell Biology, Johns Hopkins School of Medicine (Dr. Li Chen); Dept. of Medicine, V. A. Hospital, Memphis, TN. (Dr. A. Postlethwaite)

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Malaria

INSTITUTE AND LOCATION
NIAID, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
24/12	11/12	13/12

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Investigations are directed at host defense mechanisms and immunoregulation in malaria and leishmaniasis, and the possible role of the schistosomal egg granuloma in the immunopathogenesis of hepatic fibrosis. Presently, research is aimed at (1) spleen function in malaria immunity (2) regulation of intracellular growth of leishmania in human macrophages, and (3) characterization of a fibroblast stimulating factor produced in vitro by schistosomal egg granulomas. In addition, clinical studies are covered by project Z01 AI 00141-03 FY 1979.

PHS-6040
(Rev. 10-76)

Project Description:

This project investigates host defense in malaria and leishmaniasis, and immunopathogenesis of hepatic fibrosis in schistosomiasis. The studies include continuation of the program investigating the role of the spleen in malaria, as well as clinical immunology of leishmaniasis. In both cases, the motivation for this research is the belief that through gaining greater insights into basic defense mechanisms, new stratagiesmay emerge for prevention of these important protozoal diseases. Schistosomiasis research, on the other hand, investigates a model we described which provides the first molecular link between chronic inflammation (schistosomal egg granuloma) and fibroblast activation which may lead to hepatic fibrosis. The practical goal will be to consider in what pharmacologic or immunologic manner the fibrotic process could be inhibited.

Studies of splenic host defense mechanisms in rodent malaria have revealed that clearance of infected erythrocytes by the spleen is not antibody-dependent but rather determined by factors involving decreased deformability of infected erythrocytes (Wyler and Quinn) and altered splenic microcirculation (Wyler and Quinn; Wyler and Chen). Antibody protects *in vivo* in malaria by preventing merozoite invasion of RBC (Quinn and Wyler). Acute resolution of malaria is spleen-dependent. Congenitally asplenic mice as well as adult-splenectomized mice cannot overcome an acute malaria infection, but are protected from a challenge with the homologous parasite strain following rescue with chloroquine (Wyler & Oster). Reconstitution of asplenic mice with syngeneic or autologous spleen cells do not restore the protective function. These findings, taken together, clearly indicate that splenic architecture - not merely spleen cell populations - are critical in malarial host defense.

Leishmania species which are human pathogens have been sucessfully grown in human monocyte-derived macrophages (Wyler, Berman & Dwyer) and the intracellular parasite has been shown to multiply within the phagolysosomes. This model is being employed for studying the leishmanicidal effects of drugs (Wyler and Berman), as well as host factors which regulate intracellular growth of the parasite (Wyler).

Schistosomal egg granulomas, when isolated from infected mice and cultured *in vitro*, elaborated soluble substances which stimulated fibroblasts *in vitro* (Wyler, Wahl, Cheever, Wahl). This substance is distinct from soluble egg antigens (SEA), although SEA can also directly stimulate fibroblasts. The stimulating substance also can increase PGE_2, CAMP, and collagen synthesis in fibroblast cultures.

All projects are being discontinued at N.I.H. since the P.I. is leaving N.I.H. in November 1979, but will be continued in his new laboratory.

Publications:

1. Wyler, D. J., Wahl, S. M., Wahl, L. M.: Hepatic Fibrosis in schistosomiasis. Egg granuloma secrete fibroblast stimulating factor in vitro. Science, 202:435-440, 1978.

2. Wyler, D. J., Wasserman, S. I., Karchmer, A. W: Substances which modulate leukocyte migration are present in CSF during meningitis. Annals of Neurology, 5:322-326, 1979.

3. Wyler, D. J.: Leishmaniasis. In Current Therapy (Conn, H., Ed.) 1979. W. B. Saunders Co., Philadelphia, Pa.

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9. Quinn, T. C. and Wyler, D. J.: Intravascular clearance of parasitized erythrocytes in rodent malaria. J. Clin. Invert. 63:1187, 1979.

10. Neva, F. A., Wyler, D. J., and Nash, T. Curaneous leishmaniasis - a case with persistent organisms after treatment in presence of normal immune response. Am. J. Trop. Med. Hyg. 28:467, 1979.

11. Wyler, D. J., Weinbaum, F. I. and Herrod, H.: Characterization of the in vitro proliferative responses of human lymphocytes to leishmanial antigens. J. Infect. Dis., 1979 (in press).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00149-04 LPD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Physiology of Anopheles Mosquitoes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. F. Beach	Staff Fellow, LPD, NIAID
	R. M. Rosenberg	Staff Fellow, LPD, NIAID
Other:	R. W. Gwadz	Research Entomologist, LPD, NIAID

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Malaria

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
39/12	24/12	15/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project consists of a series of studies on the physiology, behavior, genetics and vector competence of anopheline mosquitoes which serve as vectors of malaria. Special emphasis has been placed on the neuro-hormonal regulation of feeding behavior, oogenesis and blood meal retention in female mosquitoes and the onset of mating behavior in male mosquitoes. The genetic and physiological basis for vector competence is being investigated in Anopheles pharoensis. The concept of antibody as a systemic insecticide is being explored using various mosquito metabolic products and hormones as antigens.

PHS-6040
(Rev. 10-76)

Project Description:

The object of this project is to investigate a wide range of physiological, behavioral and genetic parameters which influence the capacity of anopheline mosquitoes to transmit malaria. The following studies are currently underway.

1) Neuro-hormonal control of blood feeding behavior.

The demonstration in our laboratory that the biting behavior of female mosquitoes is modulated by the ovarian hormone, ecdysone, has raised the possibility that blood feeding (and consequent disease transmission) could be inhibited by providing exogenous sources of this hormone. Indeed, when ecdysone analogues are fed to female mosquitoes in their sugar meals, blood-feeding behavior is inhibited.

2) Initiation of Oogenesis.

Ovarion follicles do not develop until triggered by a blood meal. Although the midgut and ovaries are apposed, at least some of the stimuli from the engorged gut to the ovary are relayed by the brain. Using the techniques of decapitation and blood enemas, evidence now indicates that two signals are needed for the ovary to produce mature eggs. The first stimulus triggered by the ingestion of even small amounts of blood activates oocyte pinocytosis; the volume of the blood meal stimulates the brain to release a hormone which insures oocyte maturation.

3) Relationships between oogenesis and blood-meal retention.

Retention of the blood meal, as measured by the time of defecation, is a function of ovarian development. Females decapitated soon after a blood meal do not mature eggs and defecate earlier than normal. Ovariectomized females resemble decapitated females by defecating earlier than normal, but when treated with the ovarian hormone, ecdysone, these females retain blood. These and other results suggest that the blood meal initiates oogenesis, the developing oocysts produces ecdysone, and ecdysone prolongs retention of gut contents.

4) Neuro-hormal regulation of male mating behavior.

Newly emerged male mosquitoes do not mate because they can not perceive the stimuli which elicits mating behavior, the flight sounds of the female mosquito. Males detect this sound via erect hairs on their antennae. However, at emergence and for some time thereafter these hairs are recumbent on the antennal shift and do not respond to female sounds.

Antennal hair erection is triggered by the release of neurotransmitters from the nerves which ennervate the antennae. In vitro- and in vivo hair erection can be stimulated by certain α-adrenergic compounds such as L-epinephrine. However, these drugs cannot stimulate hair erection in young males.

Hemolymph transfer experiments have shown that a humoral factor is involved in the inhibition of antennal hair erection in newly emerged males. One of the candidates for this inhibitory factor is ecdysone, the growth hormone present at the time of adult emergence but absent soon thereafter. When young males are injected with ecdysone, the length of the non-erect period can be extended, there is no response to an epinephrine stimulus and the males will not mate. It appears that high ecdysone titres at emergence prevent antenna response.

5) Antibody as systemic insecticides.

Ideally, host antibodies to mosquito internal organs and secretions could enter the mosquito with the blood meal and disrupt normal function. In nature, the mosquito's host has the opportunity to form antibody only to mosquito salivary fluid. We are examining the possibility of immunizing vertebrates with antigens that could cause premature death of the biting mosquito. Testing of the antigenic potential of mosquito ovaries and midgut has already begun.

6) Genetics of vector competence of Anopheles pharoensis.

Factors which determine vector competence, particularly those which determine the susceptibility or refractoriness of mosquitoes to the pathogens they transmit, have been shown to be genetically determined. This study is designed to establish patterns of susceptibility within populations of the Egyptian malaria vector An. pharoensis. Mosquito lines susceptible or refractory to Plasmodium cynomolgi (a simian parasite closely related to the human parasite, P. vivax) are being established and maintained by single pair and mass matings. These lines will be used to determine the mode of inheritance of susceptibility, and once genetically defined these lines will be used to study some of the factors which determine susceptibility.

Proposed Course:

Investigations will be continued on the role of ecdysone in regulating a number of physiological and behavioral events in adult mosquitoes. A radio immunoassay will be employed to determine ecdysone titers in male mosquitoes. Particular emphasis will be placed on a comprehensive study of the laboratory biology and characteristics of An. pharoensis in support of on-going studies of field populations of this species in Egypt. Sera from animals immunized with ecdysone or juvenile hormone will be tested as systemic insecticides. These hormones are critical physiological modulators in mosquitoes and interference in their activity could have dramatic results.

Significance to Biomedical Research:

Anopheline mosquitoes are solely responsible for the transmission of malaria from man to man. However, factors which regulate their behavior and physiology are poorly understood. Our investigations are aimed at developing an understanding of certain critical and possibly vulnerable stages in the

life cycle of these disease vectors. Disruption of one of these events or interference in the capacity to transmit malaria could prove to have a major impact on the health on man in the tropics.

Publications:

1. Beach, R. F.: Mosquitoes: Biting behavior inhibited by ecdysone. Science (in press)

2. Nijhout, H. F. and Carrow, G. M.: Diuresis after a bloodmeal in female Anopheles freeborni. J. Insect. Physiol. 24: 293-298, 1978.

Nijhout, H. F. and Martin, S. K.: Alpha-adrenergic activity of isoproterenal in mosquito antennae. Experientia 34: 758-759, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00160-03 LPD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Cell Biology of Entamoeba, Giardia and Other Parasitic Protozoa

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	F. D. Gillin	Senior Staff Fellow, LPD, NIAID
Other:	L. S. Diamond	Head, Parasite Growth & Differentiation Section, LPD, NIAID
	T. E. Nash	Medical Officer, LPD, NIAID

COOPERATING UNITS (if any)
Dr. Andrew Plaut, Gastroenterology Div., Tufts New England Medical Center.
Dr. Pierre Daggett, American Type Culture Collection.

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Parasite Growth & Differentiation Section

INSTITUTE AND LOCATION
NIAID, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
21/12	12/12	9/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The current work is part of a study of the cell biology and cell physiology of human intestinal protozoan pathogens. Previous reports were focussed predominantly upon E. histolytica. The present report describes studies with Giardia lamblia in the following areas:

1) The role of reducing agents in promoting attachment, growth and resistance to killing by oxygen.

2) The kinetics of and requirements for attachment in a model system and the effects of host factors and chemotherapeutic agents upon attachment.

3) Interactions of G. lamblia trophozoites with complement.

4) Permeability and surface properties of cysts.

5) Isolation of new strains.

6) Antibody response in serum and secretions.

PHS-6040
(Rev. 10-76)

Project Description;

Our interests are in the cell biology and cell physiology of two important human intestinal protozoan pathogens, as well as in the biochemical and immunological aspects of the interactions of these parasites with their hosts. While previous reports were focussed predominantly on Entamoeba histolytica, the current report describes new studies with Giardia lamblia, which is increasingly recognized as a problem within the United States.

I. Growth, Oxygen-Sensitivity and the role of L-cysteine.

Maximal growth of G. lamblia depended upon the presence of L-cysteine (6-12 mM), even under N_2 atmosphere. L-cysteine appeared to be a specific growth factor. Of many compounds tested, only glutathione, N-acetyl-L-cysteine or L-cystine supported slight growth. Both L-cysteine and D-ascorbic acid also functioned as reducing agents as they delayed the killing of G. lamblia trophozoites by increased O_2 tension: ∿140 mm Hg, compared with 30 mm or less under normal growth conditions.

II. Attachment to glass

A. Giardia lamblia trophozoites show a striking tendency to attach to the walls of their culture vessel. We have observed this attachment in axenic cultures as well as with trophozoites from duodenal aspirates and from in vitro excystation.

A convenient new assay for attachment in axenic cultures was developed. It took advantage of the fact that after removal of the free cells, attached Giardia trophozoites can be detached by chilling and then enumerated in a Coulter Counter. Free swimming and attached cell populations were separated and used to initiate cultures. The doubling time of the cultures initiated with attached cells was 8.6 hours compared with 11.5 hours for cultures initiated with free cells; however the final yields were the same. Attached cells also had 2-3 fold higher cloning efficiencies. Thus, although trophozoites could detach and re-attach, the attached population as a whole, appeared "healthier".

B. As a pre-requisite to studying the process of parasite attachment in mammalian cell and organ cultures, the kinetics and requirements for the attachment of the trophozoites to glass were determined and a simple maintenance medium (MM-2) was devised.

Attachment was rapid; ∿ 75% of the maximum values were attained in 1 hour or less. Both attachment and survival were dependent upon cysteine; other thiol compounds were 30-80% as active, while ascorbic acid, a non-thiol reducing agent, was inactive. Serum (bovine or human), which also promoted attachment and survival, could be replaced by Cohn serum fraction III which consists mainly of β-globulins. Other bovine serum fractions such as Cohn II, IV, or V (albumin), as well as transferrin, β-lipoprotein, fetuin and gastric mucin were inactive. This activity was in the > 10,000 molecular weight

fraction of the Cohn III preparation. Both attachment and survival were very sensitive to pH and ionic strength: pH 6.9-7 and 200-300 milliosmoles/kg were optimal. The maintenance medium (MM-2) derived from these studies has been employed to study the effects of the chemotherapeutic agent Atabrine (quinacrine HCl) upon attachment and survival.

Secretory factors, including immunoglobulins are likely to be important in host defense against giardiasis. In preliminary studies, low concentrations (6 µg/ml) of a partially purified preparation of secretory IgA from pooled human colostrum (C/S-IgA) inhibited attachment of trophozoites. At higher C/S-IgA concentrations or with longer incubations, trophozoites were killed. These effects were observed in growth or maintenance medium in the presence of Cohn serum fraction III, but were totally prevented by 1% whole serum.

Highly purified S-IgA from another human source (p/S-IgA) inhibited attachment to a lesser extent, without killing the parasites. Heating the C/S-IgA (56 C, 20 min) reduced the attachment-inhibiting activity to the level observed with p/S-IgA. It is not known whether the S-IgA molecules have anti-giardial specificity, however binding of IgA from the C/S-IgA to trophozoites was revealed by indirect fluorescence.

III. Host Defences (complement)

Others have shown that the alternate complement pathway is activated by E. histolytica, resulting in lysis of the parasite. In contrast, we have shown that G. lamblia trophozoites do not activate complement. Killing of the parasites by rabbit hyper-immune serum, however, is strictly complement dependent.

IV. Excystation

We have confirmed the studies from Meyer's laboratory showing that: 1) excystation is promoted by a period of cyst maturation at low temperature followed by mild acid treatment and 2) cyst preparations vary greatly in their ability to excyst. We have shown that regardless of these factors, cyst populations are heterogeneous: most cysts are able to exclude fluorescent compounds which penetrate trophozoites e.g. quinacrine. Secondly, a small proportion of cysts have exposed sugars similar or identical to N-acetylglucosamine, as shown by binding of fluorescent lectins. In contrast, to date, we have not observed binding of any lectin to the trophozoite. Acid treatment of the cysts increased both their permeability to fluorescent probes and surface labelling with lectins.

V. Isolation of G. lamblia from patients

Only one strain of G. lamblia is currently established in axenic culture. Although our attempts to establish new cultures from duodenal aspirates have not been successful, we have made several useful observations:

1) Of the six media tested, TP-S-1 was the best.

2) Human serum was superior to bovine serum.

3) Trophozoites died within 1 hour if left in the duodenal fluid.

4) Only cultures from which the mucus and duodenal fluid were removed, by changing the medium, survived longer than 24 hours.

VI. Sera, parotid secretions and duodenal fluid from patients and controls (normal and hypogammaglobinemic) are being gathered (in collaboration with Dr. T. Nash). An Elisa method for determining antibody response to G. lamblia is being developed. The effects of host defenses upon (1) trophozoite attachment and survival and (2) upon excystation (as described in previous sections) will be determined.

Publications:

Gillin, F.D., and Diamond, L.S.: Clonal growth of Entamoeba histolytica and other Entamoeba in agar. J. Protozool. 25: 539-543, 1979.

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Gillin, F.D., and Diamond, L.S.: Clonal growth of Giardia lamblia trophozoites in a semi-solid agarose medium. J. Parasitol. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00161-03 LPD

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Immunochemistry of Parasitic Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: T. E. Nash, Senior Investigator, LPD, NIAID

Others: Fouad Boctor, Fogarty Fellow, LPD, NIAID
A. W. Cheever, Assistant Chief, LPD, NIAID
David Taylor, Fellow, Naval Medical Research Institute
Eric A. Ottesen, Senior Investigator, LPD, NIAID
Nasir-ud-Din, Associate in Biological Chemistry, Laboratory of Carbohydrate Chemistry, Harvard Medical School
Carl F. T. Mattern, Senior Investigator, LPD, NIAID
Wendell A. Daniel, Biologist, LPD, NIAID
Frances D. Gillin, Senior Staff Fellow, LPD, NIAID
Louis S. Diamond, Head, Parasite Growth & Differentiation, LPD, NIAID
Milford N. Lunde, Research Zoologist, LPD, NIAID

COOPERATING UNITS (if any)

Laboratory of Clinical Investigation, NIAID, Naval Medical Research Institute, Harvard University

LAB/BRANCH

Laboratory of Parasitic Diseases

SECTION

Host-Parasite Relations Section

INSTITUTE AND LOCATION

NIAID, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
22/12	17/12	5/12

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A number of studies centering around the identification and composition of schistosome antigens as well as the response of the host to parasite antigens are in progress. A series of experiments were performed in order to study the control of release of schistosome excretory-secretory (E-S) substances in vitro following incorporation of labelled N-acetyl-glucosamine. The processes involved are complex and multiple, inhibited by Na flouride, colchcine, carbachol, and secretions from other schistosomes. Damage to the schistosome tegument was associated with increased release of materials. The schistosome secretory processes are most likely dependent on energy metabolism, microtubular function and worm movement. Identification of large and small molecular weight E-S products of adult S. mansoni and B. malayi microfilaria continue. IgG ELISA antibody to a glycoprotein fraction of adult worms in infected patients was found to correlate with intensity of infection. Clinical studies involving G. lamblia trophozoites obtained from duodenal aspirates of patients as well as the specific antibody responses of the host to the parasite have been initiated.

Project Description:

A major effort of this laboratory continues to be the study of schistosome antigens. Recent investigations have concentrated on the control of release of schistosome excretory-secretory (E-S) antigens. After labelling adult schistosomes with tritiated N-acetylglucosamine, attempts were made to inhibit E-S processes by certain inhibitory procedures or substances. The metabolic inhibitors Na azide and Na arsenite resulted in the release of both large and small molecular weight products into the media. This release corresponded with sloughing of the worm's outer surface, the tegument. On the other hand, Na flouride treated worms showed decreased release of E-S materials and no tegumental loss. Worms held at 0 degrees C showed a transitory early increased release of materials. These worms were not irreversibly damaged since they were viable after rewarming. It is postulated that procedures which functionally or mechanically disrupt the tegnment result in increase loss of material. Both carboachol, an acetylcholine like substance, and colchcine inhibited secretory processes at certain time periods. Cycloheximide failed to inhibit secretory processes over the time period studied. Electronmicroscopy confirmed that Na aresenite caused tegumental sloughing at the level of the plasma membrane. Additionally, it was found that large molecular weight E-S substances inhibited the release of secretions from other schistosomes (Nash, Mattern, Daniel).

Antibodies to a glycoprotein fraction derived from adult schistosomes were measured in infected patients' sera by an ELISA test. The levels of IgG antibodies found correlated with the worm burden of the host. In contrast, antibodies to purified gut associated proteoglycan as measured by a similar ELISA test, correlated with IFA IgG antibodies to schistosome gut epitielial cells. Levels were highest in acutely infected patients and fell in the chronically infected individuals despite high levels of egg excretion. Therefore antibodies to purified fractions of worms have correlated with important and different clinical parameters (Nash and Lunde). This is distinct from the failure of clinical parameters to correlate with antibodies to crude fractions.

Studies are underway to investigate the synthesis and turnover of schistosome tegument using radioactively labelled amino sugars (Nash and Taylor).

The analysis of small and large molecular weight E-S material derived from adult S. mansoni cultured with carbohydrate precursor materials continues. Presently, emphasis is being placed on identification of small molecular weight glucosamine containing compounds.

Analysis of small and large molecular weight E-S products of microfilaria of B. malayi has begun (Nash and Ottesen).

Clinical studies involving patients infected with G. lamblia have been initiated. Headway is being made in attempts to culture the parasite from duodenal secretions. Specific antibody responses to G. lamblia are being studied in sera and other body secretions.

Publications:

1. Nash, T. E.: Antibody response to a polysaccharide antigen in schistosomiasis. I. Sensitivity and specificity. Am. J. Trop. Med. Hyg. 27: 939, 1978.

2. Nash, T. E., Ottesen, E. A., Cheever, A. W.: Antibody response to a polysaccharide antigen present in schistosome gut. II. Modulation of antibody response. Am. J. Trop. Med. and Hyg. 27: 944-950, 1978.

3. Amyx, H. L., Asher, D. M., Nash, T. E., Gibbs, C. J., Gagdusek, D. C.: Hepatic amebiasis in spider monkeys. Am. J. Trop. Med. Hyg. 27: 888, 1978.

4. Boctor, F. M., Nash, T. E., Cheever, A. W.: Isolation of a polysaccharide antigen from Schistosoma mansoni eggs. J. Immunol. 122: 39-43, 1979.

5. Nash, T. E.: Schistosoma mansoni: Pattern of release of secretory products. Experimental Parasitology. (In press).

6. Nasir-ud-din, Nash, T. E., Jeanloz, R. W., McArthur, J. W. and Gminskin, D. M.: Immunologically induced alteration in the morphology of the cervical mucus of Macaca radiata. Fertility and sterility. (In press).

7. Neva, F. A., Wyler, D. J. and Nash, T. E.: Cutaneous leishmaniasis - A case with persistent organism after treatment in presence of normal immune response. Am. J. Trop. Med. and Hyg. 28: 467-471, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00162-03 LPD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Biochemical Cytology of Host-Parasite Interactions in Parasitic Protozoa

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. M. Dwyer	Research Microbiologist, LPD, NIAID
Other:	J. Berman	Clinical Associate, LPD, NIAID
	J. Finerty	Research Microbiologist, LMI, NIAID
	M. Gottlieb	Guest Investigator*
	P. Pinto DaSilva	Head, Membrane Biology Section, LPP, NCI
	E. C. Weinbach	Head, Physiology & Biochemistry Section, LPD, NIAID
	D. J. Wyler	Senior Investigator, LPD, NIAID
	T. B. Fioretti	Chemist, LPD, NIAID

COOPERATING UNITS (if any)

* Dept. Pathobiology, School of Hygiene & Public Health, Johns Hopkins University, Baltimore, Md.

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Cell Biology and Immunology

INSTITUTE AND LOCATION
NIAID, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
48/12	36/12	12/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Aspects of the cell biology and immunology of host-parasite interactions among several intra- and extracellular parasitic protozoa are being investigated. Leishmania sp. and Trypanosoma sp. are being used as models of intracellular and extracellular parasitism, respectively. Emphasis is placed upon: 1) determining some of the basic chemical and antigenic properties of parasite surface membranes; 2) ascertaining the nature and extent of the interactions of parasite surfaces with specific host cell types; 3) defining the basic mechanisms involved in the intracellular survival and multiplication of parasites within host cells; and 4) attempts to determine the means by which parasites circumvent host immuno-defense systems. Techniques employed in these studies include: subcellular fractionation, ultracyto- and immunochemistry, electron microscopy, affinity chromatography, polyacrylamide gel electrophoresis, lectin assays, radio isotope labeling, gel immunoassays, and in vitro cell culture.

PHS-6040
(Rev. 10-76)

Project Description and Objectives:

How parasites evade host immune responses, are nourished, multiply, and eventually destroy the host are questions being asked in this investigation. In as much as all initial interactions between host and parasite occur at the level of their respective cell membranes, and understanding of the nature and physiology of these membranes is essential. It is to this end that parasite surface membrane structure, chemical composition, antigenic nature, and physiology are being investigated specifically with regard to leishmaniasis and trypanosomiasis. Further, parasite interactions with specific host cell types and immune systems are being studied to ascertain the role of parasite surface membranes in infectious disease processes.

Methods Employed:

Techniques used include: 1) fine structure (transmission and scanning electron microscopy, ultrastructural cyto- and immunochemistry, freeze fracture and -etching methods, and autoradiography); 2) subcellular fractionation; 3) analytical and preparative polyacrylamide slab gel electrophoresis and isoelectric focusing; 4) antibody and lectin binding assays; 5) radio isotope labeling and autoradiography; 6) chromatography (gel permeation and affinity); 7) qualitative and quantitative gel immunoprecipitin assays; and 8) in vitro cell culture.

Major Findings:

I. Isolation and characterization of subcellular components from Leishmania and Trypanosoma.

1. Isolation, structure, and carbohydrate-and antigenic composition of Leishmania donovani surface membranes (Dwyer).

Utracentrifuge bouyant density floatation methods were devised to increase the yield of isolated L. donovani promastigote pellicular membranes (PM). Using these methods, the PM yield (>98% purity) was increased by ∿4 fold (i.e. >15 mg protein/isolation from $5x10^{10}$ cells) over our preceeding method. These membranes had a density equivalent to ∿1.19 g/cm^3 and displayed a characteristic structural asymmetry (i.e. subpellicular microtubules remained attached to the PM inner lamina). Dissolution of the PM bilayer occurred to varying degrees following treatment with 7 different nonionic and zwitterionic detergents. The subpellicular microtubules remained insoluble to varying degrees in these detergents. Triton X-100 treatment, although only ∿50% effective with regard to its solubilizing activity, rendered most PM peptide constituents (∿40) soluble. The PM Triton extract (PM-TE) was used for subsequent antibody and lectin studies. X-ray microprobe analyses indicated the presence of relatively large quantities of sulfur and phosphorous in the isolated PM presumably representing phospholipids and sulfur containing amino acid residues.

The isolated L. donovani PM were specifically "stained" with fluorescein conjugated heavy meromyosin. These results indicate the presence of

actin or an actin-like constituent in the PM-microtubule complex.

Isolated PM were specifically "stained" for the sugars _-D-mannose (Man), _- and β-linked galactose (_-, β-gal), N-acetyl-galactosamine (NAc Gal), -glucosamine (NAcGlu), and _-L-fucose (Fuc) using eight different lectin-fluorescein conjugates. Fine structure localization results with ferritin conjugates of these lectins indicated that most, if not all, glycosylated ligands were asymmetrically oriented in the PM outer surface lamina. Detergent solubilized PM separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Periodic acid-Schiffs reagent (PAS) had ∿20 major presumptive carbohydrate containing constituents ranging in apparent molecular weight from < 1.2 to $> 28 \times 10^4$ daltons. Similar SDS-PAGE PM preparations were "stained" with various lectin-fluorescein conjugates, photographed, and subsequently stained with Coomassie Blue to determine both the characteristic specific lectin - "stained" carbohydrate - and peptide banding patterns. With the exception of a presumptive glycolipid band, all lectin - "stained" PM bands were coincidently stained for protein suggesting that most PM carbohydrates were side chain ligands on membrane glycoproteins. The lectin - "stained" PM bands had relative mobilities coincident with those obtained by the PAS method. Many PM glycoproteins were "stained" by 2 or more of the various lectins indicating compositional heterogeneity in their carbohydrate side chains. Cumulative results suggest that PM have a minimum of 20 glycoprotein constituents some of which contain Man, _- and β-Gal, NAcGal, NAcGlu and Fuc, and these are presumably oriented on the PM external surface lamina.

Antibodies were raised in rabbits against the isolated PM. These have been, and currently are being used in a variety of gel immunoprecipitin assays to ascertain the antigenic complexity of the PM. Precipitin and SDS-PAGE analyses have demonstrated the polyvalancies of these sera against the PM-TE. These anti-membrane sera have also given positive precipitin results against 7 PM mannose containing glycopeptides which were isolated from PM via lectin-affinity chromatography. Polysaccharide antigens isolated from 6 different trypanosomatid species (by Gottlieb) were specifically precipitated by anti-L. donovani PM sera. These results suggest that all of these species possess a common or cross-reacting surface membrane antigen. Further, this "common" antigen was also detected as an "exoantigen" in the sera of L. donovani infected hamsters. Sera from such animals concomitantly gave a single positive precipitin against the PM-TE indicating the presence of antibodies against some PM antigens other than the "common" polysaccharide.

2. Freeze-fracture and -etching of L. donovani PM (Pinto DaSilva and Dwyer).

Freeze-fracture and -etching studies were performed in order to ascertain further information concerning the supramolecular structure of isolated PM. Fine structure observations of such preparations demonstrated the intramembranous particle (IMP) distribution in PM. Greater numbers of

IMP were associated with the "P-" rather than the "E-face" of PM. The subpellicular microtubules (MT) remained attached to the fractured PM inner lamina. The "P-Face" IMP appeared to be aligned with respect to the attached MT; however, no structural bridge was discerned between the MT and PM inner lamina. The PM "E-face" IMP were randomly distributed. These results suggest that the MT might be attached to the PM via a inner lamina peripherally oriented membrane protein.

3. Enzyme characterization of L. donovani PM (Gottlieb and Dwyer).

In order to establish functional biochemical and physiological markers for isolated L. donovani PM a series of enzymes were investigated using standard colorimetric and radioisotopic procedures. Some of these have also been localized in the PM using ultrastructure cytochemical techniques. The enzymes activities were characterized with regard to pH and temperature optima, cation requirement, and the time course of the reaction. All enzymes examined to date have temperature optima above that used for optimum growth (i.e. 26°C). Acid phosphatase had a pH optimum of 5, was fluoride sensitive, and was as active in whole cells as in homogenates suggesting its external surface orientation. In isolated membrane fractions, this activity was enhanced 5-7 fold over that of the homogenate. No activity was present in the alkaline pH range. These results in conjunction with cytochemical localization results have demonstrated that this enzyme is indeed localized on the outer surface of the PM. This finding is of considerable significance as acid phosphatase is a lysosomal marker enzyme! These results suggest that the organism's plasma membrane is pre-adapted for life in a hydrolytic environment. Hexokinase appears not to be associated directly or at least firmly bound to the PM. 5'-nucleotidase was fluoride sensitive and was distinguished from the acid phosphatase by pH optimum and substrate specificity. Its activity was enhanced >5 fold over the initial homogenate. Cytochemical results indicate that this enzyme is PM bound. ATPase activity was not significantly inhibited by fluoride and had a pH optimum of ∿7.0. The activity was stimulated by Mg++, slightly inhibited (<20%) by ouabain, and was ∿2 fold enriched in the PM fraction over the initial homogenate. Preliminary cytochemical data indicate that this enzyme is localized on the PM inner lamina.

4. Isolation and characterization of L. donovani kinetoplast-mitochondria (Weinbach and Dwyer).

A highly enriched kinetoplast-mitochondrian (K-M) fraction was obtained from homogenates of L. donovani using various differential and sucrose gradient centrifugation techniques. The isolated K-M had a bouyant density of ∿1.22 g/Cm^3, and they characteristically possessed inner and outer membranes, cristae, and some amount of supercoiled K-DNA. The terminal respiratory metabolism of these organelles is being investigated currently with regard to characterization of their constituent enzymes and terminal electron acceptors. For details see Dr. Weinbach's report (i.e. ZO1 AI 00098-23).

5. Isolation of surface membranes from Trypanosoma rhodesiense (Finerty and Dwyer).

Methods were devised for the successful isolation of pellicular membranes (PM) from T. rhodesiense bloodstream forms. These membranes had a bouyant density equivalent to $\sim$1.176g/cm^3. They retained their structural and spacial asymmetric association with subpellicular microtubules, however, they lacked the surface coat characteristic of the intact parasite plasma membrane. Results of SDS-PAGE analyses indicated that the isolated PM contained $\sim$32 major peptides ranging in apparent molecular weight from <1.4 - 30 X 10^4 daltons. These membranes also contained a characteristic major peptide band at $\sim$5.3 X 10^4 daltons which was presumed as tubulin.

II. Host-parasite interactions in vitro.

1. Human monocyte derived macrophages (Berman, Dwyer, and Wyler).

The intracellular fate of L. donovani and L. tropica amastigotes was investigated in vitro using a human peripheral monocyte derived macrophage culture system. Results obtained using fine structure labeling methods indicated that the intracellular parasites reside and multiply within the phagolysosomal system of this host cell type. These results suggest that parasite survival is based upon resistance to host lysosomal enzyme digestion. For details see Wyler's report (i.e. #ZO1 AI 00109-07 LPD).

Proposed Course:

1. Various monospecific antibody, lectin, and radiochemical probes will be used further to qualitatively and quantitatively elucidate the nature of the intact Leishmania sp. and Trypanosoma sp. PM.

2. Specific antibody and lectin affinity chromatography and radiolabeling techniques will be used to preparatively isolate specific parasite PM constituents for their subsequent detailed chemical analyses (i.e., amino acid and carbohydrate composition).

3. Monospecific antibodies against Leishmania PM antigens will be used as probes to ascertain the presence and identity of parasite antigens on the surface of infected macrophages in vitro. Such antibodies might also be tested for their efficacy to suppress parasite growth and multiplication in cultured macrophages in vitro.

4. Lipid compositional analyses of the isolated parasite PM are planned in collaboration with a tentative postdoctoral candidate and Dr. Edgar Ribi (Rocky Mt. Lab., NIAID).

5. Studies will be pursued concerning the nature and cellular origin of parasite exoantigens present in host circulation, and their possible

effects on the state of host immune responsiveness.

Significance:

Basic research concerning the molecular and structural composition of specific protozoan parasite surface membranes and their interactions with specific host cell types might provide a basis for future immunoprophylaxis programs and regimens and, or, a more rational approach toward the development of effective delivery systems for chemotherapeutic treatment of these diseases of man.

Publications:

1. Dwyer, D. M.: Membrane interactions between Leishmania and host cells. In Schlessinger, D. (Ed.): Microbiology-1979. Washington, D. C., American Society of Microbiology, 130-134.

2. Dwyer, D. M.: Externally disposed surface membrane constituents of Leishmania donovani promastigotes. Proc. IV Int. Cong. Parasitol., Sect. F: 37-38, 1978.

3. Capron, A. and Dwyer, D. M.: The surface antigens of parasitic protozoa. In Hutner, S. (Ed.): Protozoological Actualities. Allen Press, Lawrence, Kansas. (In press).

4. Dwyer, D. M.: The recent advances in in vitro cultivation methods of trypanosomatids. In Hutner, S. (Ed.): Protozoological Actualities. Lawrence, Kansas, Allen Press. (In press).

5. Dwyer, D. M.: Isolation and partial characterization of surface membranes from the human protozoan pathogen, Leishmania donovani. Proc. Nat. Acad. Sci. USA. (In press).

6. Dwyer, D. M. and D'Alesandro, P. A.: Isolation of surface membranes from Trypanosoma lewisi bloodstream forms. J. Parasitol. (In press).

7. Berman, J. D., Dwyer, D. M., and Wyler, D. J.: Growth of Leishmania in human macrophages in vitro. Infect. Immun. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00174-02 LPD

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Culture, physiology and antigenic analysis of sexual and asexual erythrocytic malaria parasites

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	T. Shiroishi	Zoologist, LPD, NIAID
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	I. Green	Senior Investigator, LI, NIAID

COOPERATING UNITS (if any) M. Aikawa, Case Western Reserve University, Cleveland; J. D. Haynes, Dept. of Immunology, WRAIR; R. Schmidt-Ullrich, Tufts University Medical School, Boston.

LAB/BRANCH

Laboratory of Parasitic Diseases

SECTION

Malaria

INSTITUTE AND LOCATION

NIAID, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
130/12	79/12	51/12

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Animals have been successfully vaccinated against sexual and asexual malaria parasites. In addition, Plasmodium falciparum, the major malaria of man, has been grown in culture for the production of asexual stages and gametocytes. The present study will 1) Improve culture conditions for the production of large numbers of gametocytes, gametes and merozoites of P. falciparum; 2) Study of the physiology of exflagellation, fertilization and invasion of red cells by malaria merozoites; 3) Characterize structure, function and immunogenicity of surface determinants on gametes and merozoites; 4) Evaluate gamete vaccines in model systems for identification of the best antigens and adjuvants.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

1. Improvement of culture conditions for sexual and asexual parasites of Plasmodium falciparum. This will include methods for fractionation of asexual and sexual parasites and attempts to understand the controls on gametocytogenesis and development of gametocytes in culture.

2. Study of the physiology of gamete formation, fertilization and merozoite invasion of RBCs.

3. Study of structure, function and immunogenicity of antigenic determinants on the surface of extracellular sexual and asexual parasites. This will include the use of monospecific antibodies produced by hybridomas.

4. Study of immunogenicity of crude antigenic preparations of sexual and asexual stages in model systems.

Each objective is interrelated with the others: Culture will be needed for production of antigenic material for analysis of both sexual and asexual erythrocytic parasites. Structure and function of parasite determinants will be studied in the framework of parasite physiology. Successful immunization with whole parasite preparations will identify sources of more purified immunogenic materials.

Methods:

1. Continuous culture of red cell stages of Plasmodium falciparum; isolation of sexual and asexual stages; purification of merozoites and gametes.

2. Use of model systems: P. gallinaceum-chicken for study of gametes and gamete immunity; P. knowlesi-rhesus for study of mechanism of invasion and gamete immunity.

3. Use of polyspecific antibody to parasite antigens for immune precipitation; high-resolution gel electrophoresis and electrofocussing separation techniques; ^{125}I lactoperoxidase surface labelling; general metabolic labelling of all parasite components.

4. Monospecific antibody produced in hybridomas for analysis of parasite surfaces and isolation of surface determinants.

Major Findings:

1. The conversion of asexual parasites of P. falciparum to gametocytes in culture was shown to be controlled by environmental stimuli. On subculture gametocyte production ceases during a period of rapid asexual proliferation coincident with the deterioration of the health of the asexual parasites. At parasitemias above 2%, asexual parasites begin to convert to gametocytes.

This was uninfluenced by the age of the red cells in culture.

2. Using a method for measuring conversion to gametocytes, it was shown that 1 mM cAMP added to the culture medium at the end of the rapid phase of asexual growth stimulated the almost complete conversion of asexual parasites to gametocytes. Metabolities of cAMP had no influence on conversion. Theophylline, a phosphodiesterase inhibitor, stimulated gametocytogenesis in the presence of limiting concentrations of cAMP.

3. Following immunization of Balb/c mice with gametes of P. gallinaceum, spleen cells from the immunized mice have been fused with myeloma cells using polyethylene glycol. Hybrid cells grown in selective medium (HAT) have been tested for the production of antigamete antibodies using immunofluorescent antibody test against gamete preparations as an assay. Cloned lines of "hybridoma" cells producing antigamete antibodies have been selected; high levels of monoclonal antibodies were produced by growing the cloned cell lines as ascites tumors in Balb/c mice. Monoclonal antibodies have a variety of specificities for gamete antigens including internal and surface antigens of macrogametes and antigens involved in immobilization and agglutination reactions of microgametes. Using I^{125} surface labelled macrogametes certain monoclonal antibodies have been shown to precipitate a single major protein.

4. Hybridoma cell lines have been developed against merozoites of P. knowlesi. Most such hybridoma will produce antibodies which are specific for surface components of the merozoites as determined by fluorescent antibody reactions against living intact merozoites.

5. Mild digestion of merozoites of P. knowlesi with trypsin destroys the ability of the merozoites to penetrate erythrocytes. Such digestion is associated with the loss of certain high molecular weight bands on SDS polyacrylamide gel electrophoresis. These proteins may be involved in the attachment of merozoites to red cells.

6. Human serum from individuals with a high degree of naturally-acquired immunity to P. falciparum precipitates surface antigens of merozoites of P. knowlesi following I^{125} surface labelling. The precipitation of these bands is specifically inhibited by antigens of P. falciparum. This indicates that a number of antigens of P. falciparum share common specificities with the surface antigens of merozoites of P. knowlesi.

7. a) It is regularly noted that active malarial infections exert a non-specific suppressive effect on the host's immune system and may affect vaccination. We have found that monkeys with chronic P. knowlesi infections showing a rising parasitemia at the time of immunization develop little immunity to gametes after immunization with an antigen preparation containing microgametes, macrogametes and trophozoites in Freund's complete adjuvant (FCA). Chronically infected monkeys without patent parasitemia developed immunity to the sexual stages of the parasite.

b) It has been suggested that the merozoite is the most effective antigen for immunization against the asexual stages of the malaria parasite. When monkeys were immunized with P. knowlesi trophozoites (12 hours old) in FCA, they developed immunity against the asexual stages of the parasite as evidenced by low grade infections when challenged.

8. A factor has been identified in the gut of mosquitoes which induces gametogenesis in gametocytes of P. gallinaceum. Preparations containing this factor may be obtained by feeding Anopheles stephensi on saline solutions and collecting the fluid excreted through the mosquito anus during the feeding process. Preliminary chemical characterization of the active factor has shown it to be a molecule(s) of molecular weight 200-300 following fractionation on a sephadex P400 column. The factor is stable to boiling for 10 minutes but is destroyed by heating to 300°C. In chloroform/methanol extraction the factor is recovered only from the methanol fraction. The only amino acids identified in the active fraction from sephadex P400 are lysine, serine, threonine and glycine. The factor is on present evidence, a low molecular weight organic molecule without lipid properties.

Proposed Course:

1. Determine the role of cAMP in gamecytogenesis of P. falciparum in culture at the metabolic level and in relation to the phase of growth of the parasites in culture.

2. Use the methods worked out in the studies described here to obtain cultures of P. falciparum producing large numbers of gametocytes free from asexual parasites in order to conduct studies on the biology and immunology of antigens of the sexual stages of this parasite.

3. Using metabolic labels, determine the nature of the gamete proteins synthesized during gametogenesis in terms of their location in the gametes, their biological function and their role in antigamete immunity.

4. Further characterize the monoclonal antigamete antibodies produced from hybridomas and especially i) determine their ability to prevent gamete fertilization in vitro and in the mosquito and ii) isolate the target gamete antigens and determine their locations, biological functions and role in antigamete immunity.

5. Determine the chemical nature of mosquito exflagellation factor (MEF) using sephadex column fractionation, mass spectroscopy and gas liquid chromatography.

6. Use incorporation of labelled amino acids into mature schizonts of P. knowlesi to determine whether merozoites contain antigens formed during the final stage of schizont development preceeding rupture.

7. Analyze surface antigens of merozoites by immune precipitation of surface labelled and metabolic labelled antigens and characterize such antigens in relation to their role in red cell invasion and protective immunity.

8. Further characterize the monoclonal anti-merozoite antibodies produced from hybridoma cell lines and especially i) determine their ability to prevent merozoite invasion of red cells in vitro and ii) isolate the target merozoite antigens and determine their location, biological function and role in protective immunity.

9. Develop the Babesia bovis culture system for analagous studies on babesia merozoites. These babesial merozoites invade red cells in a similar manner to malarial merozoites and offer certain advantages over malarial merozoites that include long stability and availability in large numbers from culture. In addition, the probable receptor, a complement component, has been identified. We will ask the question: Will isolated, purified merozoite proteins induce protective immunity?

Publications:

1. Carter, R., Gwadz, R. W. and McAuliffe, F. M.: Plasmodium gallinaceum: Transmission blocking immunity in chickens I. Comparative Immunogenicity of Gametocyte and Gamete-containing Preparations. Expl. Parasitol., 47, 185-193 (1979).

2. Carter, R., Gwadz, R. W. and Green, I.: Plasmodium gallinaceum: Transmission blocking immunity in chickens II. The effect of antigamete antibodies in vitro and in vivo and their elaboration during infection. Expl. Parasitol., 47, 194-208 (1979).

3. Carter, R. and Miller, L. H.: A Method for the study of gametocytogenesis by Plasmodium falciparum in culture: Evidence for environmental modulation of gametocytogenesis. Bull. Wld. Hlth. Org. (in press).

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5. Carter, R. and Gwadz, R. W.: Infectiousness and Gamete Immunization in Malaria. Chapt. 17 in Malaria in Man and Experimental Animals. Ed. J. P. Krier, Academic Press (in press).

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7. Miller, L. H., Aikawa, M., Johnson, J. G., and Shiroishi, T.: Interaction between cytochalasin B-treated malarial parasites and erythrocytes. Attachment and function formation. J. Exp. Med., 149, 172-184, (1979).

8. Martin, S. K., Miller, L. H., Alling, D., Okoye, V. C., Osan, G, J. F., Osunkoye, B. O., Deane, M.: Severe malaria and glucose-6-phosphate-dehydrogenase deficiency: A reappraisal of the malaria/G-6-PD hypothesis. Lancet I: 524-526 (1979).

9. Spencer, H. C., Miller, L. H., Collins, W. E., Knud-Hansen, C., McGinnis, M., Lobos, R. A., Feldman, R. A.: The resistance factor to Plasmodium vivax in Honduras. The Duffy blood group negative phenotype. Amer. J. Trop. Med. Hyg. 27, 664-670 (1978).

10. Miller, L. H., McGinnis, M. H., Holland, P. V., Sigmon, P.: The Duffy blood group phenotype in American blacks infected with Plasmodium vivax in Vietnam. Amer. J. Trop. Med. Hyg., 27, 1069-1072 (1978).

11. Gwadz, R. W. and Green, I.: Malaria immunization in rhesus monkeys: A vaccine effective against both the sexual and asexual stages of Plasmodium knowlesi. J. Exp. Med., 148, 1311-1323 (1978).

12. Miller, L. H.: Editorial Note. Continuous in vitro cultivation of the human malaria parasite. Annals of Internal Medicine., 89, 419 (1978).

13. Martin, S. K., Miller, L. H., Hicks, C. U., David West, A., Ugbode, C., Deane, M.: Frequency of Blood Group Antigens In Nigerian Children with Falciparum Malaria. Trans. Roy. Soc. Trop. Med. & Hyg., 73, 216-218.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00184-01 LPD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Chemotherapy of Malaria: The Relationships Between Gametocytocidal, Sporontocidal and Hepatic Schizontocidal Drugs.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: R. W. Gwadz Research Entomologist, LPD, NIAID

Other: L. C. Koontz Biologist, LPD, NIAID
L. H. Miller Head, Malaria Section, LPD, NIAID

COOPERATING UNITS (if any)
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Laboratory of Parasitic Diseases

SECTION
Malaria

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
14/12	2/12	12/12

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are attempting to develop a simple rapid, inexpensive method for screening for anti-malarial drugs with activity against hepatic schizonts. We are determining if drugs with known activity against hepatic schizonts i.e. drugs which block relapses of certain human and simian malarias, will have gametocytocidal activity in the P. gallinaceum/chicken/Aedes aegypti system.

PHS-6040
(Rev. 10-76)

Project Description:

Gametocytes share certain similarities with latent exoerthrocytic hepatic schizonts in that both stages remain dormant until stimulated to undergo further development. The stimulus for gametocytes to complete development to the gamete stage is provided in the gut of a feeding mosquito. The stimulus for the exoerythrocytic parasite to complete development in the liver is unknown. The two stages also share a common sensitivity to -aminoquinoline drugs, particularly primaquin. Primaquin is the drug of choice for radical cures of relapsing malarias such as P. vivax. Therefore, drugs exhibiting gametocytocidal effects might produce a radical cure of a relapsing malaria by eliminating latent parasites from the liver. Alternatives to primaquine are desireable because of the drug's long course of therapy (14 consecutive days), because of unacceptable side effects in some individuals, and because of the high rate of drug failure against some strains of P. vivax.

Methods:

We have developed a simple screen using P. gallinaceum, in chickens which can detect drugs with gametocytocidal or sporontocidal properties by their effects on the subsequent development of the sexual stages of the parasite in Aedes aegypti mosquitoes. Gametocytocidal drugs act directly on gametocytes circulating within erythrocytes in the vertebrate hosts. Effects are irreversible and present as non-infectivity of the parasite to mosquitoes. Sporontocidal drugs affect the parasite after the parasite because extracellular and after initiation of gametogenesis within the gut of the mosquito vector. In this screen a candidate drug is administered directly to a chicken showing a patent parasitemia. One batch of mosquitoes is fed on the chicken just prior to drugging and another batch five hours later. The two groups of mosquitoes are then held 6-7 days, dissected, and their guts examined for the presence and number of developing oocysts. Drugs with gametocytocidal or sporontocidal properties effect parasite development in two ways 1) Oocyst numbers are significantly reduced, usually by over 95%; 2) Oocysts which do develop are less than 1/3 normal size at 7 days post-feeding and do not complete development. Drugs with blood schizontocidal properties have no effect on the development of the parasite in the mosquito.

Gametocytocidal and sporontocidal drugs can be further differentiated with a 2 step technique using membrane feeders. 1) Blood from a previously drug-treated chicken is washed, resuspended in normal plasma and fed to mosquitoes through a membrane. 2) Plasma from a drug-treated chicken is mixed with parasitized blood from an untreated bird and fed to mosquitoes through a membrane. Drugs with gametocytocidal properties affect the parasite in the circulation; washing (technique #1) does not restore gametocyte infectivity. Conversely, plasma from a drug treated chicken has no inhibitory properties (technique #2). Sporontocidal drugs affect the parasite only within the gut of the mosquito. Parasites

washed, resuspended in normal plasma and fed to mosquitoes are infectious. Plasma from drug-treated chickens mixed with parasites from untreated chickens inhibits oocyst growth.

Results:

A series of coded compounds provided by the Division of Experimental Therapeutics, WRAIR, as tested with the P. gallinaceum/mosquito screen. After the code was broken we found that drugs effective in producing radical cures of P. cynomolgi in rhesus monkeys, i.e. drugs which act against latent hepatic schizonts, had clearly demonstrable gametocytocidal properties in our screen.

Drugs with sporontocidal properties were less clearly differentiated. Some of these, such as pyrimethamine, appeared to have both gametocytocidal and sporontocidal effects, although pyrimethamine does affect hepatic schizonts.

Proposed Course of the Project:

This study will continue and be expanded to evaluate a wide variety of compounds provided through WRAIR. Particular emphasis will be placed on development of methods for differentiating gametocytocidal drugs from sporontocidal drugs. Protocols will be developed for the screening of newly synthesized compounds with the aim of developing new classes of hepatic schizontocides.

Significance to Biomedical Research:

A substitute for primaquine, the only available hepatic schizontocide, is considered one of the major needs for malaria therapy. However, development of new drugs has been hampered by the lack of a simple, inexpensive screen capable of evaluating a wide range of candidate compounds. We feel that the P. gallinaceum/Ae. aegypti system may function as a primary screen for hepatic schizontocides, with a capacity for evaluating large numbers of compounds including classes of drugs not yet tested against the latent tissue phases of the malaria parasite.

Publications:

1. Koontz, L. C. Jacobs, R. L., Lummis, W. L., and Miller, L. H.: Plasmodium berghei: Uptake of clindamycin and its metabolities by mouse erythrocytes with clindamycin-sensitive and clindamycin-resistant parasites. Exp. Parasitol. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00185-01 LPD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Pathogenic Protozoa: Structure, Division, Virulence Factors, and Endogenous Viruses.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: C. F. T. Mattern Senior Investigator, LPD, NIAID

COOPERATING UNITS (if any) Dr. L. S. Diamond, LPD; Dr. B. M. Honigberg, University of Massachusetts; Drs. Michael R. Spence and John K. Frost, Johns Hopkins University; Dr. Shigeko Nomura, NCI; Dr. Lawrence Abramson, NNMC, Bethesda.

LAB/BRANCH
Laboratory of Parasitic Diseases, NIAID

SECTION
Cell Biology and Immunology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
31/4	1	21/4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project (recently transferred from LVD to LPD with three personnel) is to evaluate a variety of factors which influence the virulence of pathogenic protozoa, especially Entamoeba histolytica and Trichomonas vaginalis.

Ongoing studies continue evaluating amebal viruses, selection pressures by repeated animal passage at limiting dilution, and an amebal "toxin" in the virulence of several strains of E. histolytica. Amebal extracts are toxic for mammalian cells, producing rounding and aggregation of cells in tissue culture, closely resembling the cytotoxicity of the plant lectin, concanavalin A. The former effect is inhibited by normal serum of many species. We have identified the serum component as the glycoprotein, fetuin. Fetuin and the hexosamine, N-acetyl-D-galactosamine inhibit and reverse the cytotoxicity of amebal extracts as does α-methyl-mannoside the cytotoxicity of concanavalin A. We believe the amebal toxin to have lectin-like properties. We are employing affinity chromatography to purify the "toxin" for further study.

PHS-6040
(Rev. 10-76)

Summary (continued):

Studies continue on alternate passage of relatively avirulent amebal strains in newborn hamster liver and axenic culture. We have successfully increased virulence about 1,000-fold twice with each of two strains, one having remained highly virulent in axenic culture alone for 20 months.

Project Description:

Entamoeba histolytica "toxin".

The major emphasis of this project in the past year has been in the identification of the factor in normal serum which neutralizes the cytotoxicity of crude amebal extracts for baby hamster kidney cells. The cytotoxic effect, seen only in the absence of serum, was first described by Lushbaugh et al., Medical University of South Carolina.

Amebal extracts produce rounding and aggregation of cells with dose dependent kinetics. The serum neutralizing factor has been found to be, in all probability, the serum glycoprotein, fetuin. At concentrations of 50 to 100 ug/ml, three of four commercially available fetuin preparations completely inhibit 10^5 amebal equivalents of "toxin"/ml. The purest available fetuin (GIBCO, prepared by the Spiro method, 99% pure) is the most active preparation tested, showing partial protection against the above "toxin" at 12.5 ug/ml. Other purified glycoproteins similar to fetuin, including asialofetuin, α-1 acid glycoprotein, and carcinoembryonal antigen at 100 ug/ml were ineffective in neutralizing amebal "toxin".

A number of purified IgG fractions of normal sera (mouse, rabbit, goat, human) were ineffective at 1 to 16 mg/ml against the "toxin".

Serum (>0.5%) or fetuin (50-100 ug/ml) not only completely inhibit the toxin, they also reverse the rounding and aggregation after the cells have been in that state for as many as 5 days. Such cells, so treated, reattach to the tissue culture well and divide.

Concanavalin A, a plant lectin, produces a virtually identical cytopathology with baby hamster kidney cells which toxicity is inhibited and reversed by 0.1 M α-methylmannoside and less efficiently by mannose. The amebal "toxin", however is also inhibited by a hexose, the hexosamine, N-acetyl-D-galactosamine.

The similarity in cytopathological effects of amebal extract and concanavalin A, their inhibition and reversal by N-acetyl-D-galactosamine and α-methyl-mannoside, respectively, and the evidence provided by Lushbaugh et al. that the amebal "toxin" is a protein of 25,000-35,000 daltons suggests to us that the "toxin" has lectin-like properties. This is supported by a known lectin-like, mannose inhibited, carbohydrate-binding surface protein of the free-living, but sometimes pathogenic

ameba, Acanthamoeba castellani. (Mattern, Keister, Natovitz).

Selection and maintenance of virulent subpopulations of E. histolytica.

The Rahman strain of E. histolytica has been increased 3 orders of magnitude in virulence by alternate passages for 1 week in newborn hamster liver and 1 week in axenic culture. For passage in medium, liver lesions were selected from among the largest lesions produced at limiting dilution. The first isolate, after three such passages, has remained highly virulent for the newborn hamster liver for a period of 20 months of biweekly culture in axenic medium. A repeat experiment has resulted in a second virulent Rahman line which has remained virulent for 5 months of continuous axenic culture.

Strain H-302:NIH E. histolytica has been similarly increased 100-to 1,000-fold in virulence by alternate liver passage and axenic culture. After up to 8 liver-culture passages, all such lines have rapidly declined in virulence in axenic culture alone. We are continuing the liver passages, repeating the experiment, and evaluating virulence stability of clonal lines of this strain. (Mattern, Keister).

Trichomonas vaginalis.

A collaborative project on T. vaginalis (B. M. Honigberg, Univ. of Mass.; Michael R. Spence and John K. Frost, Johns Hopkins Univ.) is pending approval of their grant in order to obtain and thoroughly characterize fresh clinical isolates of trichomonads.

Ultrastructural study of microfilaria.

An electron microscope study, paralleling light microscope observations, was undertaken on the phenomenon of antibody dependent cell adhesion between human leukocytes and Brugia maylai microfilaria. Deposition of eosinophile lysosomal material onto the microfilarial surface was demonstrated. (Weil, Daniel)

Ultrastructural study of schistosomes.

In conjunction with studies of the release of schistosome antigens by metabolic inhibitors (Na azide, Na fluoride, and Na arsenite) the kinetics of tegumental damage was followed by electron microscope study of Schistosoma. (Nash, Daniel)

Publications.

1. Mattern, C. F. T., Keister, D. B. and Caspar, P. A.: Experimental Amebiasis III. A rapid in vitro assay for virulence of Entamoeba histolytica. Am. J. Trop. Med. Hyg., 27: 882-887, 1978.

2. Mattern, C. F. T., Keister, D. B. and Diamond, L. S.: Experimental Amebiasis IV. Amebal viruses and the virulence of Entamoeba histolytica. Am. J. Trop. Med. Hyg., 28, 653-657, 1979.

3. Mattern, C. F. T., Keister, D. B. and Caspar-Natovitz, P.: Entamoeba histolytica "toxin": fetuin neutralizable and lectin-like. Am. J. Trop. Med. Hyg. (in press).

4. Mattern, C. F. T.: Structure of viruses. in "Medical Microbiology: Principles and Concepts", Samuel Baron, editor. Addison-Wesley, San Francisco (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00113-16 LPD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Studies on dengue

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Leon Rosen, Head, Pacific Research Section, LPD,NIAID

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Pacific Research Section

INSTITUTE AND LOCATION
NIAID, NIH, Honolulu, Hawaii

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project was terminated October 1, 1978

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00163-02 LPD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Field and laboratory studies on the transovarial transmission of Japanese and St. Louis encephalitis viruses by mosquitoes.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Leon Rosen, Head, Pacific Research Section, LPD,NIAID

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Pacific Research Section

INSTITUTE AND LOCATION
NIAID, NIH, Honolulu, Hawaii

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project was terminated October 1, 1978.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00164-02 LPD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Epidemiology of Infectious Disease in the Pacific Region

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: A. Dean — Pacific Center for Geographic Disease Research - Honolulu under Contract to LPD,NIAID (Contract No. No 1-A1-02228)

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Pacific Research Section

INSTITUTE AND LOCATION
NIAID, NIH, Honolulu, Hawaii

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project was terminated October 1, 1978.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00178-01 LPD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Studies of the mechanism of transovarial transmission of arboviruses by mosquitoes.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Robert B. Tesh, Staff Member, Pacific Research Section, LPD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Pacific Research Section

INSTITUTE AND LOCATION
NIAID, NIH, Honolulu, Hawaii

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project was terminated October 1, 1978

PHS-6040
(Rev. 10-76)

LABORATORY OF STREPTOCOCCAL DISEASES
1979 Annual Report
Table of Contents

PHS-NIH
Summary Statement
Laboratory of Streptococcal Diseases
October 1, 1978 to September 30, 1979

Research Progress

Bacterial Lipoteichoic Acid

A. Lipoteichoic acids (TA) are ubiquitous glycerol-phosphate polymers of Gram-positive bacteria that are primarily associated, in cell fractions, with the mesosomes. Previous studies showed large changes in total extractable LTA occurring with stages of culture growth, so we examined cells (of Staphylococcus aureus) at different culture ages by electron microscopy to determine possible changes in the number or sizes of mesosomes. No differences were found, despite 10-fold changes in LTA content. Extraction procedures were then changed from use of whole cells (old method) to use of mechanically-disrupted cells. Subsequent comparisons showed that, whereas exponentially-growing cells extracted by either method gave comparable results, whole stationary cells yielded only 10% of the LTA extractable if the cells were first disrupted. Use of the latter method (applied also to Streptococcus faecium and Bacillus licheniformis) demonstrated that the amount of total LTA was actually constant (at 1-3% dry weight of cell) throughout the cell cycle. The finding emphasizes the previously unrecognized need for complete cellular disruption prior to application of other procedures (i.e., delipidization with chloroform-methanol, which seems to fix LTA in an unextractable form), if total LTA is to be successfully extracted. The result also raises questions, as yet unanswered, concerning reasons for variation in LTA extractability according to cell age. It is possible that changes in envelope permeability, in age-associated cell wall fragility, or in dissociation of LTA from unextractable complexes with protein, are responsible, and all these possibilities will require exploration. It may also be that the variability is related to other observations on the partition of LTA among the various cellular fractions. It was found that if cells were frozen before protoplasting (an essential first step in fractionation), LTA shifted to the periplasm and the shift was greater in exponential than in stationary cells. Mechanical disruption alone caused a shift of LTA to the soluble fraction; this was enhanced by the absence of added magnesium ion, particularly in exponential cells. Continuing studies will utilize standard procedures (unfrozen cells, added magnesium, Braun mechanical disruption) now shown to be optimal, in exploring LTA turnover, reactions of LTA with mesosomal proteins and lipids, and intracellular enzymes involved in LTA degradation. (Huff, Theodore, Popkin)

B. By use of a hot phenol extraction instead of previous methods, purified LTA without protein, nucleic acids, hexoamines, or cell wall contaminants, was obtained form Staphylococcus

aureus. The material contained only glycerol and phosphate (1:1), d-alanine, glucose, and 5 fatty acids, of which the C_{15} species was predominant. Immunization of rabbits with this LTA coupled to methylated bovine serum albumen produced antiserum that was specific for the glycerol-phosphate backbone moiety of the LTA; antisera raised previously against whole cells, mesosomes, membranes, or hot water and cold phenol extracts, were cross-reacting and did not detect LTA because of specificities directed primarily against contaminating proteins. The specific antiserum was of high activity (2.5 mg antibody/ml) and was employed to verify chemical data indicating the presence of LTA primarily in the mesosomal fraction of cells and its absence in culture fluids. It will now be used (as IgG) to localize LTA by immuno-electron microscopy in thin sections of intact cells, which was not possible with previous antisera. In addition, the sensitive serologic detection of LTA has been made quantitative by development of a radioimmunoassay method for use in ongoing studies of LTA structure, biosynthesis, and functional role in the bacterial cell. (Theodore; Greenberg and Kalica, LID, NIAID)

Legionnaires' Disease Organism

The morphology and ultrastructure of the cultured bacillus responsible for Legionnaires' Disease, first investigated by us last year, was reaffirmed by subsequent and repeated electron microsopic examinations. The cultured organism, as well as that first correctly reported in human lung sections in conjunction with our CDC collaborators (Am. J. Clin. Pathol. 71, 43-50, 1979) is a simple double-membrane-enveloped rod ultrastructurally characteristic of Gram-negative bacteria. No organelles such as pili or flagella are present, and no layer representing peptidoglycan between outer and inner (cytoplasmic) membrane was visualized. Except for large lucent granules or inclusions ultrastructurally typical of polyhydroxybutyrate, no unusual intracytoplasmic features were found. The furrowing type of cell division seen is that of Gram-negative bacteria. These features, which were reported at the International Symposium of Legionnaires' Disease (CDC, Atlanta, November 1978) and subsequently published in its proceedings (Ann. Int. Med. 90, 642-647, 1979) were confirmed, for the most part by findings of other investigators, that were also presented at the meeting. No new or different ultrastructural information has been reported since. The ultrastructural features do not contribute to an explanation of the pathogenicity or virulence of this bacterium. (Cole, Popkin, LSD; Chandler et al, CDC)

New Erythrocyte-Adherent Bacterium Infecting Man

A middle-aged white male, splenectomized 25 years earlier, developed arthralgias, anorexia, weight loss, and episodes of chills and fever associated with petechiae and tender nodular purpuric lesions of the feet. The history, especially in

relation to contact with animals, arthropods, or other similar illnesses, was non-contributory. Blood cultures, serologic tests for infectious agents, and other laboratory examinations, were negative. However, direct examination of blood smears revealed Wright- or Giemsa-staining, Gram-positive, bacteria-like bodies adherent to red cells; similar bodies, better revealed by a silver stain, were found in large numbers extracellularly in biopsies of the cutaneous lesions and in the bone marrow. Inoculation of embryonated eggs, tissue cultures, and 5 species of normal and splenectomized animals produced no results. Samples of peripheral blood, furnished to us in subsequent consultation with the clinician (Dr. G. L. Archer, Medical College of Virginia), were examined by electron microscopy in negative stains and after sectioning. We also cultured samples aerobically and anaerobically in several media. All cultures remained sterile, but electron microscopy showed short rod-shaped bacteria on red cell surfaces, but not within. No destruction or alteration of the cells was evident. The bacteria were typically of Gram-positive nature by internal features (mesosomes), septate division, and structure of the cell wall--with the exception of an additional unique feature of an outer membrane 7.5-8.0 nm wide surmounting the wall. After several episodes of the clinical features described, each relieved temporarily by vancomycin and cephalosporins but recurring with reappearance of the red cell-associated bacteria, the patient was cured by chloramphenicol and the bacteria disappeared. Negative serologies, lack of culture in artifical media or animals, and the Gram-positive ultra-structure, indicated that the bacterium was not Bartonella bacilliformis or any other previously known red cell-asscciated prokaryote. Gram-positive bacteria adherent to red cells and causing disease in humans have not been reported, and this case (New Eng. J. Med., in press) represents the first instance of what must be a rare condition caused by a bacterium that has yet to be cultivated, classified, and examined for mode of patho-genicity. (Cole, Popkin, LSD; Archer, Medical College of Virginia)

Streptococcal Erythrogenic Toxins

Our prior studies established that a non-lysogenized strain (64 x 402) of a Group A streptococcus, contrary to extant dogma, could produce an erythrogenic toxin (ET). Four antigenically distinct ETs (A, B, C, and D) are known, but only ETB (identified by monospecific antisera and standard reference strains) was produced by 64 x 402. The question raised was the location of the genetic coding for the production of ETB. No evidence of plasmids (either by isolation procedures or by loss of ETB production after exposure to "curing" acridine dyes) was found in 64 x 402. Its DNA (as well as that from a strain producing both ETs A and B) failed to transform a competent Group H streptococcus to ETB^+, although a streptomycin resistance marker was transferred. These results appeared to rule out either extrachromosomal or chromosomal DNA as the site of genetic information for ETB production. Another possibility was the presence in 64 x 402 of

a defective, and therefore undetected, phage; but recent attempts to prove this by recombination after infection of 64 x 402 with a virulent mutant phage and examination of progeny, were unsuccessful. The location of the ET coding thus remained obscure--not only for ETB, but also for the other toxins. In continued exploration, strain 64 x 402 was lysogenized by a phage from a strain producing ETs A and C-with resultant production (in addition to ETB already present) of ETC but not A. We then attempted to lysogenize $^{+}_{52}$ other Group A strains that were ETB^{-} with 4 phages from ETB^{+} strains. Only 26 strains were sensitive to the phages, only 8 were lysogenized, and none of these now produced ETB. Present information thus suggests that production of ETs A and B is not related to lysogenizing phages, but production of ETC may be; technical problems could be responsible for some nondefinitive but suggestive findings, and the subject requires further study that is under way. Adjunct studies did not reveal production of ETs A, B, or C by streptococci of Groups C (7 strains) or G (8 strains), despite reported clinical studies associating such streptococci with infection and an erythematous rash in humans. (Colon-Whitt, Cole)

Interactions of Genetically Competent Bacteria with DNA

Competent Haemophilus influenzae cells interact very specifically with DNA (Scocca, Poland, and Zoon; J. Bacteriol. 118: 369-373, 1974). It has recently been found that competent H. influenzae cells recognize specific base sequences in DNAs (Sisco and Smith; PNAS 76: 972-976, 1979). One study (Ceglowski, Fuchs, and Soltyk; J. Bacteriol. 124: 1621-1623, 1975) showed that naturally occurring glucosylated DNAs (derived from T-even bacteriophages of Escherichia coli) competed poorly, if at all, with homologous transforming DNA in a system using competent Streptococcus sanguis (strain Challis) cells as recipients. This result resembled the one obtained with H. influenzae cells and was at variance with previous data from this laboratory. Therefore, the reactions were investigated in more detail, with the following results. T-even phage DNAs reacted except for expression, with competent S. sanguis (strain Wicky) cells in the same fashion as homologous DNA. The reactions examined were (1) DNAse-sensitive DNA binding, (2) DNAse-resistant DNA binding, and (3) degradation of DNA by competent cells. From an analysis of the initial rates for the above 3 reactions, it was concluded that T-even glucosylated DNAs reacted at much slower rates, which is why they did not compete with homologous DNA for transformation. The rates were inversely related to the amount of diglucosylation; for example, the most extensively diglucosylated DNA (T6 DNA; 70% glucosylated) reacted the slowest. T2 DNA, the next most extensively diglucosylated (5%) reacted next slowest, and T4 DNA (0% diglucosylated) reacted the fastest of the three. However, its rate was still slower than that of Wicky DNA. Although these data do not suggest that specific base sequences are also recognized by competent S. sanguis cells, other data to be developed, may.

Reactions of competent S. sanguis cells with DNAs derived from HeLa cells, T5 bacteriophages, and Bacillus subtilis, were also poor. Study of such reactions is continuing in our efforts to define the mechanisms of DNA binding, uptake and dispositon during genetic transformation of bacteria. (Ranhand)

Plasmids of Spiroplasmas

Spiroplasmas (motile helical mycoplasmas infecting plants, insects, and ticks, and capable in some instances of producing disease in embryonated eggs and rodents) are poorly understood metabolically and pathogenetically. All carry one or more of 3 viruses, but other possible extrachromosomal nucleic acid elements were previously unknown. We examined 12 spiroplasma strains from diverse natural sources by dye-buoyant density gradient centrifugation followed by electron microscopy and agarose gel electrophoresis. Ten strains contained covalently closed circular (CCC) DNAs ranging in size from 1.0 to greater than 12 megadaltons molecular weight, and each strain carried from 2 to at least 6 size classes of these circular molecules. The presence, sizes, or number of size classes per strain do not correlate with either the known carriage or state of active production of the viruses, and the CCC DNAs are therefore not considered to be viral genomes. Since no host phenotypic traits have yet been associated with the presence of any CCC DNAs, these latter are currently believed to be crytpic plasmids such as those often found in bacteria. No systems of genetic transfer among spiroplasmas have been developed; future work will be directed to this aspect, as well as to definition of phenotypic markers, effects of plasmid curing techniques, development of mutants, rapid methods of detecting and sizing plasmids in spiroplasma surveys, and more precise physical characterization of the plasmids already discovered. (Ranhand, Mitchell, Cole, Popkin)

Viruses of Spiroplasmas

The survey of old and newly-acquired strains of spiroplasmas (helical motile mycoplasmas) for carriage of one or more of the 3 morphologically distinct viruses previously described, was continued by electron microscopy (EM) and plaquing methods. Cumulative data indicate that all strains, regardless of source, carry at least one virus, and there are some differences in their distributions. Spiroplasmas from bees and flowers carry only the rod-shaped virus, SpV1; those from corn stunt disease, ticks, or Drosophila carry SpV1 or SpV3 (polyhedral virus, short tail) or both; and isolates from citrus stubborn disease or otherwise identified as Spiroplasma citri, may carry 1, 2, or all 3 viruses--including SpV2 (polyhedral virus, long tail) which is not found in the other groups of spiroplasmas. SpV2 has not been isolated, propagation and characterization of SpV3 was previously described, and emphasis this year was on study of SpV1 that was first

propagated only late last year. EM showed that more than 50% of spiroplasmas carry SpV1 representatives, and 8 new isolates showing host range differences but no host modification or restriction, were made from spiroplasmas of bees, corn, and citrus. One bee isolate studied in detail has particles of characteristic size (230-280 by 10-15 nm) with buoyant densities of 1.39 g/cm^3 in CsCl and 1.21 g/cm^3 in Metrizamide gradients. Its nucleic acid is DNA and appears by EM to be double-stranded and linear, but confirmation is needed. Antiserum to this isolate neutralized other isolates equally well. Virus infectivity is also sensitive to chloroform, ether (somewhat), some detergents, temperatures above 60 C, or 4 C or lower (if prolonged), and pH below 6 or above 9. It is not affected by nonionic detergents, Genetron, DNAase, RNAase, or drying. Growth studies showed a single-hit infection, an adsorption constant of 2.55×10^{-9} cm^3/min, a 60-90 min latent period, release of 10 progeny viruses per cell by 6 hrs, and some delayed cell death not attributable, as in many other phage-host systems, to viral-induced lysis. Host cultures can be selected for resistance to one SpV1 isolate, and are also resistant to all other SpV1 isolates. This result, the inhibition of different isolates by a single antiserum, the obtention of isolates with these common properties from different host strains, and the apparent absence of host modification, suggest that SpV1 common in many diverse spiroplasmas may be identical. Further work will examine possible SpV1 group characteristics, characterize the DNA, classify the viral proteins (of which there appear to be 7 to 9), and investigate the intracellular state of this virus and its genome. (Liss, Cole, Popkin)

Streptococcal Hemolysin (Streptolysin S)

Streptolysin S, an extracellular protein that is the most powerful membranolytic agent known, is non-antigenic and of uncharacterized importance in the pathogenesis of streptococcal infections. We found it to exist as an inactive intracellular precursor in streptococci of Group A, C, and G, but not in B. In Group A, it is membrane-associated; highest titers were found in nephritogenic strains, where the greatest amounts occurred in membrane fractions and the highest specific activity in mesosomes. The precursor was previously shown to be activated by vortexing with glass beads in the presence of RNA-core, and recent work showed improvements in results by use of small beads, and beads washed with 60% acetic acid, and phosphate buffer. Although detergents alone fail to activate precursor, it can be activated (to a very labile form) by vortexing with glass beads in the presence of a nonionic detergent and then stabilized by addition of RNA-core. The precursor is neither activated nor degraded by streptococcal proteinase, but this enzyme inactivates active hemolysin--thus suggesting a possible role in regulation of hemolysin production. Current emphasis is on purfication and characterization of both precursor and active hemolysin by use of a lipid carrier, chromatography in organic solvents, transfer to a synthetic nucleotide carrier, and affinity column chromatography. (Calandra, Theodore)

Administrative, Organizational, and Other Changes

The number of research projects increased by one to a total of 7, with the return to the Laboratory of Dr. Gary Calandra from detail to LCI. Dr. David Williamson's appointment as Visiting Investigator through the Intergovernmental Personnel Act expired in February 1979, and he returned to the State University of New York at Stoneybrook to resume academic duties. The appointment of Dr. Daniel Moynet as Visiting Fellow from France was renewed to February 1980. During the summer, Ms. Jodi Abramowitz (COSTEP; New Jersey) and Ms. Maria Velazquez (Minority Biomedical Science Student Program; Ponce, Puerto Rico) received training and experience in microbiological techniques from Dr. Alan Liss (Senior Staff Fellow) while participating in our project on spiroplasmas and their viruses. It is anticipated that Dr. Liss, having completed two years here in his present appointment, will move to the Rocky Mountain Laboratory in another capacity in October 1979. No other changes occurred or are forecast. The two positions lost last year by reassignment and retirement have not been restored, and it is expected that a third position (Dr. Liss) will also be lost because of increasingly severe last-minute and unanticipated cuts in personnel ceilings imposed by OMB. The total loss to LSD brought about by Institute distribution of such cuts is disproportionate (18%) and will result in an authorized strength of only 14 positions, of which only 5 are occupied by doctoral level investigators. The future of the Laboratory is in some doubt, since the Laboratory Chief will retire mandatorily in September 1981 and the Institute leadership is meanwhile strengthening bacteriological research only at RML while maintaining only routine support of LSD in the interim. As a result, new directions of reasearch, except as may be engendered to a minor extent by new findings in current projects, cannot be expected and the prospects for bringing in new blood seem dismal. Nevertheless, we shall attempt to continue, and complete as possible, the basic microbiological research projects already in progress.

Honors and Awards

R. M. Cole

Member, Editorial Board, Infection and Immunity.

Member, Study Group on Viruses of Mycoplasmas and Spiroplasmas: Subcommittee on Bacteriophages, International Committee on Taxonomy of Viruses.

J. M. Ranhand

Invited participant, 23rd Wind River Genetics Conference (Estes Park, Colorado), June 12-15, 1979.

D. Moynet

Invited participant, 23rd Wind River Genetics Conference (Estes Park, Colorado), June 12-15, 1979.

A. Liss

Invited seminar, University of Montana, Missoula, June, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00001-18 LSD

PERIOD COVERED
October 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Biochemical Studies on Staphylococcal and Streptococcal Membranes, Cell Walls, and Mesosomal Vesicles.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. Huff	Scientist Director	LSD, NIAID
Other:	T. S. Theodore	Res. Microbiologist	LSD, NIAID
	T.J. Popkin	Chemist	LSD, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Streptococcal Diseases

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long range purpose of this project is to study the biochemical processes involved in the growth of bacterial cell wall and membrane and the coupling of these processes to DNA replication during cell division. The present goal is to determine the functional role of lipoteichoic acid in Staphylococcus aureus and other Gram-positive bacteria. Lipoteichoic acids are polymers of glycerol phosphate found intracellularly closely associated with plasma membranes and with membranous bodies known as mesosomes. A minor component of the lipoteichoic acid fraction, lipoteichoic carrier, has been implicated by others in the synthesis of cell wall teichoic acid. The function of the remainder of the lipoteichoic acid (LTA) is not known and this is the area of current interest. Present studies are directed at: 1) determining total intracellular LTA content during growth; 2) intracellular location of LTA in bacteria other than S. aureus; 3) determining specific mesosomal protein-LTA interactions.

Project Description:

Lipoteichoic acids are antigenic glycerol phosphate polymers found intracellularly closely associated with membranous organelles known as mesosomes in Staphylococcus aureus. The purpose of this study has been to determine the function of intracellular lipoteichoic acid (LTA). Lipoteichoic acid was isolated from chloroform-methanol extracted cells by treatment with hot aqueous phenol and separated on Sepharose 6B into peak I (micellar LTA) and peak II (non-micellar LTA) after treatment with DNase and RNase. We have previously reported large changes in the observed LTA level in S. aureus during growth. Since LTA is always closely associated with isolated mesosomes from this organism, we tested whether large changes in the number of mesosomes could also be observed during growth. Cells of S. aureus (Lafferty strain) were harvested at different stages of growth, negatively stained with phosphotungstic acid and examined in the electron microscope. No changes were observed in the number or size of mesosomes during growth, while 10-fold changes in LTA content were observed. This result prompted us to examine our extraction procedure. We found that undisrupted exponentially growing cells gave full yields of LTA as compared to disrupted cells. However, undisrupted stationary cells yielded only 10% of the LTA obtained in cells disrupted by shaking with glass beads. No differences were observed in the total intracellular LTA yield whether cells were disrupted in the Braun homogenizer with glass beads or by protoplasting with lysostaphin.

Previous studies had also shown large variations in the amount of LTA extracted from Streptococcus faecium and Bacillus licheniformis during growth. Experiments were run to determine if disruption was necessary to completely extract LTA from these organisms. In S. faecium (ATCC 9790) the LTA yield (measured as peak I material from Sepharose 6B columns) for stationary cells went from 0.6% (of the dry weight of the cells) in undisrupted to 1% in disrupted cells. Throughout the growth cycle the LTA level, as determined in Braun-disrupted cells, remained constant at 1.0 to 1.4% of the dry weight of the cells. The yield of LTA from early logarithmic undisrupted cells was higher (2.2%) than disrupted cells (1.4%) probably as a result of enzymatic destruction of LTA during Braun disruption. In B. licheniformis (ATCC 9945) the LTA yield from 18 hour cells went from 0.3% in undisrupted to 1.8% in disrupted cells. During growth the LTA level, as determined in Braun disrupted cells, remained at 1.8 to 2.8% of the dry weight of the cells. The highest level of LTA from undisrupted cells was 1.8%.

These results show large changes in the extractability of LTA from Gram-positive organisms dependent on the growth state of the organism. In all the species studied, the level of LTA obtained from undisrupted stationary cells was very low. After disruption, the level of LTA was very high. The high levels obtained after disruption, the constancy of these high levels during growth, and

the independence of the level on the method of disruption (mechanical versus enzymatic lysis) argue that these figures represent total LTA.

These results also point to the need for complete disruption of an organism as a necessary first step in establishment of the intracellular LTA content. Once whole cells have been treated with chloroform-methanol followed by hot aqueous phenol, the LTA appears to be fixed in the residue and not extractable.

It is not known what changes occur at the onset of exponential growth to make the intracellular LTA more extractable from undisrupted cells. This could be due to: 1) an increase in the permeability of the cell walls to LTA as the cells start to divide or, 2) the dissociation of LTA from an unextractable protein-LTA complex at the onset of cell division. It seems unlikely that mechanical disruption would break such a complex. If this complex was cleaved enzymatically, it would have to occur within the mesosome during protoplasting since LTA added during protoplasting does not exchange with mesosomal LTA. Although peak I LTA occurs predominantely in the mesosome and peak II LTA occurs predominantly in the periplasm, the extractability of these two substances from S. aureus (Lafferty) increases and decreases coordinately during growth. It seems unlikely that mechanical disruption could cause a protein-peak I LTA dissociation in mesosomes and also a similar protein-peak II LTA dissociation in the periplasm. A much simpler explanation is that the cell wall of an exponentially growing cell is more permeable to both peak I and II LTA. Another possibility is that cell wall disruption can occur during extraction of exponential cells with chloroform-methanol and hot phenol but this does not happen with stationary cells.

Determination of the intracellular location of LTA has been difficult and is controversial. In S. aureus (Lafferty strain) the LTA is predominantly in the mesosomes in cells protoplasted in the presence of magnesium, 20% NaCl and lysostaphin. If cells are frozen before protoplasting there is a shift of peak I LTA to the periplasm. This shift is 20% in stationary cells and 60% in exponential cells. Braun disintegration of either frozen or fresh cells also causes the LTA to shift into the 100,000 x g supernatant fraction. Omission of magnesium results in an even greater shift of LTA into the soluble fraction. This is particularly true in exponential cells where Braun disintegration in the absence of magnesium yields 90% of the LTA in the soluble fraction. Similarly, when B. licheniformis (ATCC 9945) cells were frozen and Braun disintegrated in the absence of added magnesium ion, none of the LTA could be recovered in the membrane or mesosome fraction at any stage of growth. When S. faecium frozen cells were Braun disintegrated in the absence of added

magnesium ion about 50% of the LTA was recovered in the mesosomal and 20% in the supernatant fraction. These ratios were constant at all growth stages. The peak II LTA varied from 0.1% of the dry weight of the stationary cells to 2% in late exponential cells and was primarily in the 100,000 x g supernatant fraction of Braun disintegrated frozen cells without magnesium. Planned are studies on LTA in B. licheniformis and S. faecium using fresh cells, magnesium, and Braun disintegration.

An attempt was made to determine if the protein found at much higher levels in mesosomes than in membranes was associated with LTA during SDS-gel-electrophoresis. P^{32}-labeled mesosomes and membranes were treated with SDS-buffer and run on slab gels. Duplicate portions of the gels were stained for protein in one sample and exposed for radioautography in the other. LTA was found to run as a diffuse band in a region free of protein bands. Thus if LTA is attached to a protein in the mesosome this complex is dissociated by SDS.

The ubiquitous presence of lipoteichoic acids in Gram-positive organisms, the location of micellar (peak I) LTA external to the bacterial membrane (in mesosomes and periplasm) and the presence of non-micellar (peak II) LTA in the periplasm and extracellularly, point up the importance of these LTA's as bacterial antigens. We have previously shown the presence of LTA antibodies in the sera of staphylococcal endocarditis patients. Recently, the lipoteichoic acids of Streptococcus mutans have been implicated in the formation of dental plaque. The present study demonstrates large changes in extractability of LTA from Gram-positive bacteria during growth. The need for complete disruption of these organisms for extraction of LTA has been established. Using these new methods, high levels of LTA (1-3% of the bacterial dry weight as peak I LTA and 1-3% as peak II LTA) have been measured at all stages of growth. Lipoteichoic acids are thus major end products of bacterial growth in Gram-positive organisms.

Planned and ongoing work includes: 1) determining if LTA is a cellular end product or if there is LTA turnover; 2) testing mesosomal protein and lipid to see if it arises from old or newly synthesized membrane in pulse-labeled experiments; 3) characterizing enzymes involved in LTA degradation; 4) characterizing the nature of the interaction between LTA and mesosomal protein and lipid; 5) continued study on the intracellular location of LTA in S. faecium and B. licheniformis.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00002-14 LSD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Studies on the Nutrition, Physiology and Ultrastructure of Staphylococcus aureus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: T. S. Theodore Res. Microbiologist LSD, NIAID

COOPERATING UNITS (if any)
Bureau of Biologics, FDA (B. Fraser)
Laboratory of Infectious Diseases, NIAID (H. Greenberg, A. Kalica)

LAB/BRANCH
Laboratory of Streptococcal Diseases

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.9	0.9	2.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Distinct features of most Gram positive bacteria are the presence of lipoteichoic acid and a membrane system consisting of a plasma membrane and mesosomes. The purpose of this project is to relate membrane structure with function using Staphylococcus aureus as the model test system. The major emphasis is to define the function (or functions) of mesosomes which contain all of the lipoteichoic acid present in the cell. To corroborate the chemical localization of lipoteichoic acid in mesosomes by immunochemical methods, an antisera specific for the polyglycerol phosphate protion of the molecule was prepared. This antisera failed to react with ribitol teichoic acid and gave one precipitin band with deacylated lipoteichoic acid and cardiolipin. Passive hemagglutination inhibition tests showed the exclusive localization of lipoteichoic acid in mesosomes; verifying our previous chemical findings. Also, preliminary data on a radioimmunoassay procedure for the quantitation of lipoteichoic acid is presented.

Project Description:

Morphologically, the most distinct feature of Gram positive bacteria are the mesosomes which arise from the membranous spetum and precede crosswall formation. The most distinct chemical feature of isolated mesosomes is the presence therein of almost all of the cell's lipoteichoic acid (LTA: a glycerol phosphate polymer containing a phosphatidyl glycolipid). LTA interacts strongly with divalent cations, participates as a carrier molecule in cell wall synthesis, and acts as a regulator of autolytic activity. Also, it is immunologically active and binds to mammalian cells. Using chemical, enzymatic and serological methods, as well as subcellular fraction techniques, the overall objective of this study was to ascertain whether mesosomes are functional organelles.

1) Immunological studies with staphylococcal LTA

Subcellular fractionation and chemical analysis have shown that most of the LTA of Staphylococcus aureus in the cell resides in the mesosomal portion of the membrane. However, the location within the cell is not precise, since it is possible that during fractionation procedures, some movement, reattachment or degradation of the LTA can occur. Therefore, a more direct approach to this problem would be by immunochemical localization of the LTA in intact cells. Initial attempts to prepare antisera for this purpose, using whole cells, mesosomes, membranes, or hot water and cold phenol extracts of LTA, unsuccessful. Multiple precipitin bands were detected by Ouchterlony technique, but, the specificity of these antisera appeared to be directed toward the protein portion of the antigens rather than the polyglycerol-phosphate backbone. These antisera failed to react with hot phenol extracted LTA, deacylated LTA, and deacylated cardiolipin. Recently, using LTA extracted with hot phenol and coupled to methylated bovine serum albumin, we succeeded in preparing an antisera specific for the backbone portion of LTA. This antigen contained no protein, nucleic acids or hexosamines. Glycerol and phosphate were present in a 1:1 ratio and the only amino acid detected was d-alanine. The preparation was devoid of any cell wall contaminants adn the only carbohydrate present was glucose. Of the five fatty acids detected, C_{15} was the predominant species. (In collaboration with B. Fraser, BOB, FDA) This antiserum, when tested by immunodiffusion against LTA extracted by hot water, or by hot and cold phenol, gave single precipitin bands that showed lines of identity among the several extracts. It also reacted with deacylated LTA and cardiolipin. No precipitin bands were observed with ribitol teichoic acid. Immunoelectrophoretic analysis showed that the antigen migrated to the anode and reacted strongly, forming only one precipitin band. Crossed and rocket immunoelectrophoresis were also used to

further verify the specificity of this antiserum: over a wide range of antigen concentrations, only one precipitin band was detected. Quantitative precipitin analysis showed that 1 ml of antisera contained 2.5mg of antibody that could be precipitated with 100 ug of antigen. Passive hemagglutination inhibition tests on the various subcellular fractions verified our chemical analyses that most of the LTA was present in the mesosomes. Mesosomal titer (reciprocal of dilution) was 1024, periplasm, 16; membrane, 2; and cytoplasm, 0. No LTA was detected in any of the culture fluids. Deacylated LTA and cardiolipin titers were 256 while ribitol teichoic acid failed to react. At present, we have prepared the IgG fractions of normal and immune sera and will now attempt to localize LTA in thin sections by immunoelectron microscopy, using the horseradish peroxidase enzyme system.

Although the evidence is still indirect and pending on the outcome of the immunoelectron microscopy, chemical and serological data support the localization of LTA in mesosomes. Since these organelles are most preminent at the time of cell division, arise from the membranous septum, precede crosswall formation, and contain LTA, it is quite probable that they play an active role in the biosynthesis of LTA, cell wall and membrane.

The planned course of this project will include immunoelectron microscopic localization of LTA in intact cells; characterization of the LTA structure; and biosynthesis of LTA and its function as it relates to the mesosome.

2) Radioimmunoassay for the Determination of LTA

Serological methods used so far are extremely sensitive for the detection of small amounts of LTA; however, they are only semi-quantitative in comparison to chemical analysis. The drawbacks of chemical analysis are that they are difficult, time consuming, and require much larger samples. For this reason, we attempted to develop a radioimmunoassay (RIA) method for the quantitative determination of LTA. Microtiter plates were coated with varying dilutions of antigen and then reacted directly with radiolabeled IgG^{125}. By this method it was possible to detect quantitatively between 10 and 100 nanograms of LTA. Present studies are directed toward refining this procedure and increasing the sensitivity of the assay. (in collaboration with H. Greenberg and A. Kalica, LID, NIAID)

3) Characterization of Rotaviral Proteins

Simian rotavirus (SA-11) was selected for the study of rotavirus polypeptides since it grows well in cell culture and possesses a hemaglutinin associated with its outer capsid. CsCl density

gradient centrifugation yielded two peaks of rotavirus. The first peak contained mostly single capsid particles and yielded 5 polypeptides on phosphate buffered SDS polyacrylamide gels. The second peak contained almost exclusively double capsid particles and exhibited 8 polypeptides on polyacrylamide gels. The molecular weights ranged in size from 52,000 to 118,000 daltons. The 3 polypeptides present in the second peak are assumed to be associated with the outer capsid of the simian rotavirus. The phosphate buffered continuous gel system used in this study appears to be comparable to the more commonly employed discontinuous tris-glycine gel system and the reported resolution of fewer bands in the former gel system was probably due concentration differences of double capsid virus rather than the gel system employed. (In collaboration with A. Kalica; LID, NIAID)

Publications:

Kwon-Chung, K. J., Bennett, J. E., and Theodore, T. S.: *Cryptococcus bacillisporus* sp. nov. Int. J. Syst. Bact. 28, 616-620, 1978.

Kalica, A. R., and Theodore, T. S.: Polypeptides of simian rotavirus (SA-11) determined by a continuous polyacrylamide gel electrophoresis method. J. Gen. Virol. 43: 463-466, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00004-19 LSD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Electron Microscopy of Bacteria in Relating Structure and Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. M. Cole	Chief	LSD, NIAID
Other:	T. J. Popkin	Chemist	LSD, NIAID

COOPERATING UNITS (if any)
See following page for cooperating units.

LAB/BRANCH
Laboratory of Streptococcal Diseases

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	0.3	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project provides support, through preparative techniques (negative staining, thin sectioning) and subsequent examination, for Laboratory and Collaborative microbiological studies requiring transmission electron microscopy. Objects of ultrastructural study include mycoplasmas and their viruses; streptococci and their bacteriophages; staphylococci; bacilli; gram-negative bacteria; fungi; and various cell fractions thereof, examined by means of cytochemistry and immunoelectron microscopy.

Cooperating Units:

Dept. of Biochemical Sciences, University of Tampere, Finland (E. Jansson)

Laboratory of Clinical Investigations, NIAID (Clinical Mycology Section; K. J. Kwon-Chung)

Laboratory of Infectious Diseases, NIAID (Mycoplasma Section; J. G. Tully)

Office of the Scientific Director, NIAID (R. Repaske)

Leprosy and Rickettsia Branch, Virology Division, Bureau of Laboratories, CDC, Atlanta (C. C. Shepard)

Division of Infectious Diseases, Medical College of Virginia, Richmond (G. L. Archer)

Laboratory of Microbiology and Immunity, NIDR (B. M. Chassy)

Pathology Division Bureau of Laboratories, CDC, Atlanta (F. W. Chandler)

Project Description:

Objectives are to provide, as required, electron microscopy facilities in support of other projects in the Laboratory; and to conduct such original and in depth studies of the ultrastructure of microorganisms as may be engendered by collaborative beginnings or by independent approach to problems.

1. New strains of spiroplasmas examined for viruses (SpV) included 1 S. citri (Iran: SpV3+), 2 corn stunt (Rio Grande, Chen, and Jamaica, Eden-Green: both SpV1+), 1 Drosophila hydei (Oishi: SpV3+), 1 bee (AS576C Davis: SpV1+), 1 flower (G1 Davis: SpV1+). Aggregate evidence from repeated EM examinations of over 50 strains indicates that bee and flower spiroplasmas carry only virus SpV1; tick, corn stunt, and Drosophila spiroplasmas carry either or both SpV1 and SpV3; and citrus stubborn disease strains (S. citri) carry any of the 3 viruses alone or in double or triple combination, and is the only spiroplasma group in which SpV2 is found. Continued monitoring of all strains carried in our collection reaffirmed the erratic occurrence from passage to passage, of massive virus production; and revealed several instances of production of viruses never before observed in large numbers in that particular strain. The stimulus for sudden virus production is still unknown.

New emphasis on propagation and characterization of SpV1 (Z01 AI 00007-5 LSD) required extensive EM monitoring of spiroplasmas as possible donors or indicator strains; frequent examination of plaques, washes, inocula, and infected broth cultures to confirm the nature of the virus produced in 8 new SpV1 isolates; and constant EM checks on stages of SpV1 purification. In addition, many Kleinschidt-type spreads of SpV1 DNA were examined and photographed in efforts to ascertain molecular size and conformation.

2. Stages of attempts to isolate and purify the internal filaments of spiroplasmas were followed by EM. Clean preparations of filaments have not yet been obtained, although several agents (Non-Idet P40, Triton X-100, Zwitterionic detergents) disrupted cells and gave good yields of crude filaments.

3. Other mycoplasmas examined, both by negative stains and sections, included 4 human isolates from Finland (Dr. E. Jansson) and 3 rod-like viruses derived therefrom, a new species of Acholeplasma (72-043: Dr. Tully), a new mouse mycoplasma (RIII-4: Dr. Tully), and flower spiroplasma BNR1 growing in chick amniotic fluid (Dr. Tully).

4. Bacteriophages examined after induction of parent strains, or during stages of purification, included several from streptococcal Groups A and P (Ms. Colon-Whitt), B (Dr. Calandra), and H (Dr. Moynet). Others were coliphage lambda from an in vitro system of DNA packaging (Dr. Repaske: Z01 AI 00132-12 OSD) and several new phages isolated from Lactobacillus casei (Dr. Chassy, NIDR).

5. Preparations of the Legionnaires' Disease Organism were reexamined and photographed, and reports prepared. (See Publications)

6. Facilities for spreading films of purified nucleic acids (Kleinschmidt technique) were developed and applied by Mr. Popkin, and used extensively to examine SpV1 spiroplasma virus nucleic acid (Dr. Liss: Z01 AI 00007-5 LSD), DNA and restriction endonuclease fragments thereof, from a Group H streptococcal phage (ø42) and some group A streptococcal phages (Dr. Moynet: Z01 AI 00006-8 LSD), and newly-discovered plasmids of spiroplasmas (Dr. Ranhand: Z01 AI 00006-8 LSD).

7. Cocci examined included Group B streptococci in stages of protoplasting by a cell wall-active enzyme, mutanolysin (Dr. Calandra: Z01 AI 00181-01 LSD); staphylococci at various stages of growth to ascertain age-related changes in number of mesosomes per cell (not found) (Dr. Huff: Z01 AI 00001-18 LSD); staphylococci after treatment with various lipid solvents to determine effects on cell wall structure (not found) and purified staphylococcal lipoteichoic acid (Dr. Theodore: Z01 AI 00002-14 LSD); and

staphylococci stained by immunolabelling after sectioning in unsuccessful attempts to find the intracellular location of lipoteichoic acid (Dr. Theodore, Mr. Hochberg: Z01 AI 00002-14 LSD).

8. Preparations of Mycobacterium leprae, in various stages of purification from armadillo livers and after different treatments (e.g., trypsin) were examined in thin sections for differences in structure of cell walls and surfaces. The results were inconclusive, and more highly purified and concentrated samples have not yet been received (Dr. Shepard, CDC).

9. Samples of blood showing by Giemsa and positive Gram stains, bacteria-like bodies adherent to erhthrocytes, were received from Dr. G. Archer (Medical College of Virginia). The blood came from a middle-aged white male with a history of splenectomy, arthralgias, anorexia, weight loss, chills, sweats, and fever, and purpuric lesions on the feet. The presumptive bacteria were also found in the skin lesions and in bone marrow. Electron microscopy, by both negative staining and sections, showed short rods adherent to but not within, erythrocytes; the rods had, with the exception of a unique outer membrane, the ultrastructural features of a Gram-positive bacillus. They could not be cultured, either here or in Richmond; the non-cultivability, the Gram-positive characteristics, and negative serological tests, ruled out known red-cell affecting bacteria such as Bartonella, Haemobartonella, Eperythrozoon, Anaplasma, and Grahamella. The patient responded, in several episodes, to vancomycin and cephalosporins, but relapsed and was finally cured by chloramphenicol. The causative organism appears to be a new bacterium, and its description and the case report will be presented in the literature. (See Publications)

10. A wild-type melanin-producing strain of Cryptococcus neoformans and a pigment-less mutant--both grown on standard malt agar and on an agar stimulating melanin production--were supplied by Dr. Kwon-Chung (LI, NIAID) for comparisons of the presence and cellular location of the pigment. Thin sections, showed the wild-type cells to have a much thicker and layered cell wall and a sub-membranous location of granules (absent in the mutant) assumed to be melanin. However, the study is incomplete, and may require use of cytochemical methods to confirm the nature of the granules.

Publications:

Archer, G. L., Coleman, P. H., Cole. R. M., Duma, R. J., and Johnston, C. L., Jr.: Human infection with an unknown erythrocyte-associated bacterium. N. E. J. Med. (in press)

Chandler, F. W., Blackmon, J. A., Hicklin, M. D., Cole, R. M., and Callaway, C. S.: Ultrastructure of the agent of Legionnaires' disease in the human lung. Amer. J. Clin. Path. 71: 43-50, 1979.

Chandler, R. W., Cole, R. M., Hicklin, M. D., Blackmon, J. A., and Callaway, C. S.: Ultrastructure of the Legionnaires' disease bacterium. A study using transmission electron microscopy. Ann. Int. Med. 90: 642-647, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00005-14 LSD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Studies on Bacteriophages and Genetics of Streptococci

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Colon-Whitt	Microbiol.	LSD, NIAID
	R. M. Cole	Chief	LSD, NIAID
Other:	G. B. Calandra	Medical Officer	LSD, NIAID

COOPERATING UNITS (if any)
Laboratory of Parasitic Diseases, NIAID (T. Mercado)

LAB/BRANCH
Laboratory of Streptococcal Diseases

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.1	1.4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long range goal of this project is to study bacteriophages and genetic exchange (mainly lysogenic conversion, transduction and transformation) in streptococci, and to investigate how such mechanisms may affect the pathogencity and immunology of these organisms. Currently we are searching for the nature and site of the genetic coding for a rare, Group A, non-lysogenic, erythrogenic toxin (ET) producing strain. We have attempted 1) to isolate a defective prophage by phage-genetic recombination, 2) to induce ETB production in ETB$^-$ strains by lysogenization with phages from ETB$^+$ strains, and 3) to find toxin production in a selected number of Group C and G streptococci. Other studies concern extracellular product of Pseudomonas fluorescens which causes morphological changes and lysis in strains of Trypanosoma cruzi. We are trying to identify the factor and assess its significance.

Project Description:

1) Erythrogenic toxin of a non-lysogenic Group A streptococci

Erythrogenic toxin (ET) or streptococcal pyrogenic exotoxins (SPE) are extracellular proteins produced by a number of Group A streptococci. Four distinctive antigenic types (A, B, C, and D) have been described and lysogeny has been considered essential for their production. We have been studying a rare, non-lysogenic strain (64 x 402) which produces ET-B toxin and, upon lysogenization or vegetative infection with a phage (ø) isolated from strain C203U (producing ETs A and C), synthesizes ETC in addition to ETB, but not ETA. These results suggested that lysogeny may not be essential for the production of all toxins, and in strain 64 x 402 the genetic coding for ETB could be (1) an integrated portion of an incomplete prophage genome or (2) an integral segment of the streptococcal chromosome or (3) a transduced fragment or (4) an integrated plasmid or transposon or (5) a free plasmid or transposon. These hypothesis were tested and some of the results were presented in the last annual report. In summary, we were unable to induce production of ET (by a) transformation [using strains 64 X 402 Sm^R (ETB) and NY5 Sm^R (ETA and B) as DNA donors and Group H strain Wicky as DNA recipient] or by (b) vegetative infection of Group A and C non-toxinogenic strains with phages isolated from strains producing ETs A, B, or C. No plasmids or transposons were found and treatment of 64 x 402 cells with acridine orange did not inhibit synthesis of the ETB toxin.

In recent studies we have attempted (1) to induce synthesis of ETB in a number of ETB^- strains by lysogenization with phages isolated from ETB^+ strains and (2) to isolate a defective phage from strain 64 x 402 by infection and recombination with a virulent phage (øV203U). In (1), 52 Group A strains were tested for sensitivity to 4 phages isolated from ETB^- strains. Plaques or lysis were observed on 50% of the tested strains. Colonies were picked from the center of the lytic area streaked on P-agar plates through 5 consecutive passages; and then tested for lysogenicity by induction with Mitomycin C and by sensitivity to the homologous phage. Lysogenic colonies were obtained from only eight strains. Numerous attempts to isolate true lysogenic colonies from the other strains were unsuccessful. The eight lysogenic strains were grown for 24 hours in 10 ml dialysate media, centrifuged, and the supernatants were filtered, concentrated 100x in Amicon cells and tested for ETB production by the Ouchterlony test against monospecific ETB antitoxin. None synthesized detectable ETB.

For isolating a defective phage, both non-lysogenic parent 64 x 402 and lysogenized strain 64 x 402 (øAT203U) were used. Eight

strains (including C203U, 64 x 402 and 64 x 402 (øAT203U) were used as phage indicators. The test strains [64 x 402 and 64 x 402 (øAT203U)] were grown in P-broth to log phase. The cultures were divided into two aliquots. One was non-induced, the other was induced by adding Mitomycin C (for 15 min) or by U. V. irradiation (for 60 sec). The cells were centrifuged and resuspended in fresh P-broth. One ml of øVAT203U (virulent mutant) was added at a MOI of 10, the tubes were incubated for 3 hrs, centrifuged and 0.1 ml of øAT203U antisera or normal sera was added to 0.9 ml of the supernatant. After 30 min incubation, 0.01 ml of the supernatant was dropped on P-agar plates previously seeded with log phase cultures of the indicator strains. Controls were supernatants of 4 hr cultures of both strains with and without phage antisera. After overnight incubation the plates were examined for plaques. There were no plaques on strains C203U or 64 x 402 (øAT203U) in samples with or without phage antisera. Samples without phage antisera plaqued in four of the indicator strains, following the same lytic pattern as øAT203U. Samples from Mitomycin C induced, with and without phage antisera, plaqued in strain K56. These plaques were picked, the phage was propagated to high titers (10^9 pfu/ml) in K56 and compared for lytic and immunological patterns with øAT203U. Both were identical. Unsuccessful attempts were made to propagate this phage in K56 in the presence of different concentrations of AT203U phage antisera. These results indicate that the phage obtained was øAT203U. This phage is very virulent for strain K56, and needs higher concentrations or longer exposure to specific phage antisera to inactivate it and prevent it from plaquing in this strain.

Recently, there has been increased interest in the production of ET by Group C and G streptococci. Several clinical cases have been reported in which only streptococci of these groups have been isolated. We studied a selected number of strains (7 Group C and 8 Group G) from our collection for production of ETA, B, and C by the method previously described. There was no evidence of ET synthesis in any of the strains.

Our failure to detect ET_B production in cells lysogenized with phages isolated from ETB^+ strains, or in Group C and G strains, indicates that either these strains do not produce the toxins or they are produced at such low levels that we need a more sensitive method of detection. Recently the Elisa test has been successfully used by other investigators. In future work we will focus on the use of more sensitive methods for toxin detection in studying the phage-cell relationships in ETB^- producing strains.

2) Screening for a Group B muralysin

There is no satisfactory method for lysing Group B streptococci.

Such a method would be highly desirable. In collaboration with Dr. G. Calandra (LSD) we investigated the possibility of a phage muralysin in Group B streptococci comparable to the phage associated lysin found in Group C streptococci. To that purpose we propagated 8 Group B phages to high titers, (10^8 -10^9 pfu/ml). The lysates were tested for lytic activity against Group B type Ia, Ib, Ic, II, and III strains, using different buffers, at different pH, with and without a reducing agent added. No muralysin was found. This project was terminated when Dr. Calandra found another source of an effective lysin.

3) Lysis of Trypanosoma cruzi by Pseudomonas fluorescens

In collaboration with Dr. T. Mercado (LPD) we are currently studying the effect produced by P. fluorescens on T. cruzi. It was observed that this gram negative, motile rod appears to be attracted to the kinetoplast-flagella site, where it attaches, eventually causing morphological changes of the parasite. Filtered and concentrated (10x) supernatants of nutrient broth cultures of the bacteria caused the same effect, with complete lysis of the parasite within 10 min after exposure. Supernatants of sonicated cells have some effect but not to the extent of culture supernatants, which indicates that the factor is mainly extracellular. Its peak of activity is found in stationary phase cultures (48 hrs). Its activity is not inhibited by heating (56 C for 30 min), freezing and thawing, or trypsin digestion, but is inhibited by boiling for 5 min. Concentrated dialysates of the supernatants retained their lytic activity, which indicates that its molecular weight is less than 12,000. Future work will be directed to identifying the extracellular factor and further investigation of its activity in vivo.

We consider these observations to be significant not only in terms of the general biology and physiology of the parasite but because of their possible impact on parasite chemotherapy and/or immunology.

Publications:

Colon-Whitt, A., Whitt, R. S., and Cole, R. M.: Production of an erythrogenic toxin (streptococcal pyrogenic exotoxin) by a non-lysogenized Group A streptococcus. In Parker, M. T. (ed.): Pathogenic Streptococci. Chertsey, Surrey; Reedbooks Ltd., 1979,pp. 64-65.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00006-08 LSD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Competence Development and Genetic Exchange Mechanisms in Streptococci.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Jon M. Ranhand	Sr. Scientist	LSD, NIAID
Other:	Daniel Moynet	Visiting Fellow	LSD, NIAID
	W. O. Mitchell	Biologist	LSD, NIAID
	T. J. Popkin	Chemist	LSD, NIAID
	R. M. Cole	Chief	LSD, NIAID

COOPERATING UNITS (if any)

Macromolecular Biology Section, LBV (F. DeFilippes)
Z01 AI 00126-06 LBV

LAB/BRANCH
Laboratory of Streptococcal Diseases

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.05	1.2	0.85

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The project as originally defined, "to study the mechanism by which competent group H streptococci take up deoxyribonucleic acid (DNA) molecules from their surroundings," is continued. I examined further the interaction between competent Wicky cells (Streptococcus sanguis) and homologous or heterologous, native or denatured, DNAs. DNAs that contain chemical modifications, such as alpha or beta glucosylation, or both, as seen in some bacterial viruses, interact with competent cells at a slower rate. This observation explains, in part, the reasons why certain DNAs do not compete with homologous DNA in transformation (Ceglowski, Fuchs, and Soltyk, J. Bacteriol. 124: 1621-1623, 1975). In addition to the above, I showed the effect of temperature on the competent-cell DNA reaction. I also showed that the amount of DNA bound to competent cells depends upon (1) the amount applied, (2) the source, and (3) the time for interaction. These results further our understanding of the competent-cell DNA reactions.

Project Description:

Part I

The object of this work is to study the competent Streptococcal cell-DNA reaction. Only competent cells are physiologically capable of interacting with DNA molecules and four intereactions are defined. (1) DNA binding to competent cells in a DNAse-sensitive fashion; this binding is thought to be on the cells' surface, (2) DNAse-resistant DNA binding; this binding is thought to be inside the cells' cytoplasm, (3) the production of acid-soluble products (ASPs) from the DNA that has reacted with competent cells; this reaction is thought to be due to specific competent cell associated nucleases, and (4) genetic recombination and expression of homologous DNA (transformation).

Reactions (1) and (2) above are essentially completed by 10 min, at 37 C; reaction (3) continues to increase beyond 60 min with some DNAs and with other DNAs it is completed by 60 min. These reactions are measured by using radiolabeled DNA.

The first 3 reactions above have now been shown to have specific temperature requirements. Reaction (1) occurs optimally between 30 and 42 C; reaction (2) has a temperature optimum of 30 C; reaction (3) has no optimum between 25 and 42 C. However, between 37 and 42 C, there was a change in slope in the Arrhenius plot. This indicates that 2 or more nucleases are responsible for ASP formation. From Arrhenius plots of the data the respective activation energies (AE) were calculated.

Reactions (1) and (2) above occurred with AEs of 23 KCal/mole; reaction (3) had an AE of 46 KCal/mole, reflecting a summation of 2 or more nucleases. AEs of 23 KCal/mole suggest enzyme mediated reactions. Therefore, DNA binding to competent cells, which was thought previously to be a physical process (electrostatic), does not occur at o C and is enzymatic.

It was also observed that when DNA was added to competent cells at concentrations below saturation (less than 2 ug/ml) the amount of DNA that was (a) cell-bound, (b) DNAse-resistant, and (c) degraded to ASPs was independent of the concentraiton. For example, when DNA was applied at concentrations between 0.12 to 1.8 ug/ml, about 24% was cell-bound, 9% was DNAse-resistant, and 70% was degraded (results for T 7 phage DNA). It was shown that DNA preparations do not contain a fraction of reactive molecules. It appears that competent WE4 cells can control, in some unknown way, the amount of DNA that they will react with.

In reciprocal experiments, where a nonsaturating concentration of DNA was added to various concentrations of competent cells, again, essentially a constant fraction of the DNAs was: cell-bound, DNAse resistant, degraded.

The initial rates for the above reactions varied and depended upon the DNA source. In the present study, the rates (molecules of DNA/cell/min/ ug DNA added) in descending order were: Wicky DNA > T7 DNA > E. coli B DNA > T7 DNA > T2 DNA > T6 DNA.

T even phage DNA is glucosylated. The glucose residues are covalently attached to hydroxymethyl cytosines. Seventy percent of T6 phage DNA is diglucosylated; 5% of T2 phage DNA is dislucosylated; and 0% of T4 phage DNA is diglucosylated. It appears, from their order of reactivity, that the more diglucosylation of the DNA, the slower it reacts. In general, glucosylation may cause the DNA to interact at a slower rate. This observation explains why T-even phage DNAs compete poorly with homologous DNAs for transformation.

The rate limiting reaction for transformation is the DNAse-resistant DNA binding step. This slow rate was also observed with labelled DNA. Reactions (1) and (3) above occur 2 to 7 times faster than reaction (2), depending on the DNA used.

This study also showed that the amount of DNA that was bound per cell depended upon the amount of DNA applied. The same was true for the other reactions. At DNA concentrations below saturation, the amount of DNA that was cell bound varied from 0.5 to 4 molecules/cell; the amount of DNA that became DNAse resistant varied from 0.1 to 1 molecule/cell; and the amount of DNA that was degraded varied from 0.6 to 7 molecules/cell. At DNA concentrations above saturation, the amount of DNA that was bound to cells varied from 3-33 moelcules, the amount of DNA that became DNAse resistant varied from 0.7 to 5 molecules, and the amount of DNA that was degraded varied from 3 to 75 molecules. At low DNA concentrations as well as high DNA concentrations the amount of DNA that was bound and taken up at 10 min equaled the amount that was bound and taken up at 30 min. In other words, these reactions were completed by 10 min, at 37 C.

From the above data, it has been calculated that competent cells can take up in a DNAse resistant form almost 10% of their total DNA content, and can bind up to 50% of their total DNA content.

(These calculations assume that S. sanguis Wicky cells contain 1 x 10^9 daltons of DNA.) Therefore, if a competent Wicky cell is 0.5 um to 1 um in diameter, then in 1 min, at 37 C, a single cell can take up a molecule of a length 8 to 17 times its own diameter. A remarkable feat!

I also showed, for the first time, that heat denatured (single stranded) DNA is bound to competent cells in a reaction analogous to native (double stranded) DNA. Calcium ion is essential. In addition, it was also shown by competition experiments that competent cells contain specific binding sites for denatured DNA.

It is concluded from the work above that competent cells react to a given concentration of DNA in such a way that they only take up a fraction of it. How this is accomplished is yet unknown.

I will continue to examine the competent-cell DNA reactions as well as continue studies on the physiology and enzymology of competence.

Part II

Mr. W. O. Mitchell and I isolated satellite DNAs by dye-buoyant density gradient centrifugation of cell lysates from 10 of 12 strains of the helical motile mycoplasmas known as spiroplasmas. (See also Projects Z01 AI 00007-5 and Z01 AI 00004-19 LSD). Electron microscopy (T. J. Popkin and R. M. Cole, LSD) and agarose gel electrophoresis revealed that the satellite materials were composed of 2 to 6 classes, per strain, of covalently closed circular (CCC) DNA molecules ranging in molecular weights from 1.0 to at least 12 x 10^6 daltons. No functions are yet known for these extrachromosomal elements. Accumulated indirect evidence suggests that they are not viral genomes, and they are considered to be cryptic plasmids. Presumptive plasmids, though of larger sizes and also without demonstrated functions, have been reported in a few other mycoplasmas, but were previously unknown in spiroplasmas. Future efforts will be directed to clarification of size classes and their distributions among these and other spiroplasmas, to possibilities for transfer among strains, and to studies of antibiotic resistances and other phenotypic traits that may aid in elucidating the functions of these plasmids.

Dr. Moynet continued studies of streptococcal phages. ø42, a temperate phage of lambdoid morphology, was propagated in a Group H streptococcus (Streptococcus sanguis: strain Wicky). By contour lengths from electron microscopy (EM) and by summation of the sizes of fragments generated with Hind III restriction endonuclease separated by agarose gel electrophoresis, ø42 nucleic acid was confirmed to be a linear double-stranded DNA of molecular weight 24.1 x 10^6 daltons. Limited treatment with E. coli Exonuclease III produced molecules with 5' single-stranded ends; the molecules subsequently circularized, providing the classic evidence of terminal repetition of nucleotide sequences. EM of DNA after denaturation and reassociation showed double-

stranded artificial circles, thus giving evidence of circular permutation of the genome. Comparisons of contour lengths of such circles with the length of the intact linear molecule indicated the extent of the terminal repetition to be between 15% and 20% of the genome. Treatment of the shortened duplex DNA resulting from exposure to Exonuclease III, by restriction enzymes Hind III (8 cuts in the intact molecule) and Pvu II (20 cuts), produced fragments which were of equal intensities and all present after gel electrophoresis. In controls of adenovirus 2 DNA, which is neither terminally redundant nor circularly permuted, a number of terminal bands disappeared after similar treatment. The evidence not only indicates the presence of permutation in ø42 DNA, but also suggests that its location in the molecule is random.

The ø42 DNA was also treated with several other restriction enzymes (Xba I, Sal I, Sac I, Pst I, Msp I) and the fragments ordered by gel electrophoresis to initiate physical mapping of the genome. In conjunction with A. Colon-Whitt, DNAs of several lambdoid phages of Group A streptococci were examined; these were of approximately similar molecular weights ($24\text{-}26 \times 10^6$ daltons) and, after treatment with several of the above restriction enzymes, showed gel patterns of fragments that were similar to one another but different from those of similarly treated phage DNAs of Group H phages. Comparisons of streptococcal phages and their DNAs, on which little information exists, will be continued as time permits. (In collaboration with F. DeFilippes, LBV)

Publications:

Ranhand, J. M.: DNA binding and uptake by competent Streptococcus sanguis Wicky cells. In Glover, S., and Butler, L. O. (eds.). Transformation 1978. Cotwsold Press, Oxford, 1979 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00007 - 04 LSD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Viruses of Spiroplasmas and Mycoplasmas

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. M. Cole	Chief	LSD, NIAID
	A. Liss	Sr. Staff Fellow	LSD, NIAID
Other:	W. O. Mitchell	Biologist	LSD, NIAID
	T. J. Popkin	Chemist	LSD, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Streptococcal Diseases

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.3	1.8	2.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project aims to characterize the nature, distribution, and ecologic importance of viruses carried by spiroplasmas. Recent studies of the rod-shaped virus SpV1 show single-hit infection kinetics, an adsorption constant of 2.55×10^{-9} cm^3/min, a latent period of 60 to 90 min, and delayed host cell death unrelated to virus-induced lysis. Virus infectivity survives non-ionic detergents, Genetron, nucleases, heterologous viral antiserum, and drying; but is sensitive to other detergents, chloroform, ether (to some extent), homospecific antiserum, pH <6 or >9, and temperatures >60 C or (if prolonged) 4 C. Eight new isolates of SpV1 from spiroplasmas from bee, corn, and citrus diseases showed host range differences but no evidence of host modification or restriction. All were equally sensitive to specific antiserum raised against the original isolate from a bee spiroplasma. Viral DNA by electron microscopy appears to be duplex and linear, but its size, precise conformation, and other characteristics are under continuing study.

Project Description:

The object of these investigations continues to be a description of the distribution, host range, biological and physico-chemical characteristics, and possible roles of viruses found in the helical mycoplasmas known as spiroplasmas.

Three virus groups, now designated as SpV1 (rod-shaped), SpV2 (polyhedral, long-tailed), and SpV3 (polyhedral, short-tailed) are known from electron microscopic observations. Propagation and characterization of SpV3 isolates was previously reported. SpV2 has not yet been isolated nor propagated. Emphasis this year was on further characterization of SpV1, which was initially propagated only late last year.

The original SpV1 isolated, designated SpV1/KC3(BC3), was made by plaquing of filtered culture supernate from one spiroplasma (KC3) that kills honeybees, on lawns of another honeybee strain (BC3). Extension of prior electron microscopic studies showed that 50% of 25 spiroplasmas from diverse natural sources carry SpV1 particles. Additional isolates of SpV1 were obtained, by use of filtered washes of lawns grown on solid media, from spiroplasmas cultured from moribund honeybees (3 isolates from 3 strains), from corn stunt disease (2 isolates from 2 strains), and citrus stubborn disease (3 isolates from 3 strains). Host range studies of the 8 isolates revealed distinct differences, but the presence of classic host modification and restriction systems has not been shown. On the other hand, spiroplasma host strains or derivatives selected for resistance to one SpV1 isolate were also resistant to all other SpV1 isolates tested to date, suggesting other mechanisms for resistance such as lack of surface receptors or failure of adsorption or of nucleic acid injection for other reasons. Antiserum to purified SpV1/CK3(BC3) was obtained by immunization of rabbits, and was of the same efficiency in inactivation (determined by plaque assay) of other SpV1 isolates as in inactivation of the homologous isolate. Aggregate data from plaquing and electron microscopic studies are being incorporated into a manuscript on the distribution of all 3 viruses among the increasing numbers of spiroplasmas cultured from such diverse sources as diseased plants, healthy flowers, sick bees, ticks, and other arthropods.

Physico-chemical studies of SpV1/KC3(BC3) previously showed that the purified 230-280 by 10-15 nm particles have buoyant densities of 1.39 g/cm^3 in cesium chloride and 1.21 g/cm^3 in Metrizamide gradients. Chemical and radiolabelling studies indicated its nucleic acid to be DNA. Recent electron microscopic studies of both aqueous and formamide spreads of the extracted DNA suggest that it is probably double-stranded and linear, but consistent

peculiarities in the preparations examined prevent a definitive description at present of its precise size and molecular conformation. Polyacrylamide gel electrophoresis of disaggregated virions showed 7 to 9 presumptive structural proteins, and calculations from virion buoyant densities indicate that approximately 23% of the particle should consist of nucleic acid. Infectivity of the virus particles was found to be sensitive to specific antisera, chloroform, some detergents, temperatures above 60 C or prolonged storage at 4 C, and pH outside the range of 6 to 9. There was moderate sensitivity to ether, and insensitivity to non-ionic detergents (Non-Idet P40, Triton X-100), Genetron, antisera to SpV3, DNAase, RNAase, and drying.

Growth studies of SpV1/KC3(BC3) on the original propagating host strain BC3, are being described in a manuscript in preparation. Infection followed a single-hit model, with a particle adsorption constant of 2.55^{-9} cm^3/min. At a low multiplicity of infection (1.0 or less), a 60 to 90 minute latent period was followed by an extended period of gradual release of progeny virus. Six hours after infection, each infected cell had released 10 progeny viruses, and there was no concomitant cell death nor lysis; however, at a much later time in the infectious cycle, there is death of infected cells which appears to be secondary and limited. Short range goals of this portion of the study include study of the intracellular state of this virus which is not detectable within infected cells by electron microscopy.

Other aims include further analysis of SpV1 DNA and proteins, as well as identification of group characteristics of SpV1 isolates. Other recent studies of this Laboratory (see Project No. Z01 AI 00006-8 LSD) have shown a range of small to moderate-sized (1.0 to 12 x 10^6 daltons) circular DNAs in 10 of 12 spiroplasmas--all of which were carrying one or more viruses, and some of which were actively producing a virus at time of examination. However, the sizes of these presumptive cryptic plasmids, the numbers of size classes found, and their distributions by strain, do not correlate with virus carraige or production, and the available evidence suggests that the circular molecules do not represent viral genomes.

These studies furnish information essential to determining the nature, and ultimately the importance, of new viruses that occur commonly in a widespread group of recently recognized and poorly understood mycoplasmas. Both viruses (which appear similar to bacteriophages) and newly-found plasmids may be expected to play roles in the host ecology similar to those of bacteriophages and bacterial plasmids, but the existence and influence of their effects in spiroplasmas remain to be elucidated.

Publications:

Cole, R. M.: Mycoplasma viruses. In Laskin, A. I., and LeChevalier, H. A. (eds.): CRC Handbook of Microbiology, ed. 2. West Palm Beach, CRC Press Inc., 1978, Vol. II, pp. 683-690.

Cole, R. M.: Mycoplasma and spiroplasma viruses: Ultrastructure. In Barile, M. F., and Razin, S. (eds.): The Mycoplasmas. New York, Academic Press, 1979, Vol. 1, pp. 385-410.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00181-01 LSD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Isolation, Purification, and Characterization of Toxins and Inflammatory Factors of Streptococci.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Gary B. Calandra	Medical Officer	LSD, NIAID
Other:	T. S. Theodore	Res. Microbiol.	LSD, NIAID
	A. Colon-Whitt	Microbiol.	LSD, NIAID
	T. J. Popkin	Chemist	LSD, NIAID

COOPERATING UNITS (if any)
Arthritis and Rheumatology Branch, NIAMDD (Dr. R. Wilder)

LAB/BRANCH
Laboratory of Streptococcal Diseases

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	1.3	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this work is to identify the toxic factors of streptococci, especially Group A, that play a role in streptococcal pathogenesis. The major emphasis is to purify and characterize the hemolysin, streptolysin S (SLS). Using the cellular precursor to SLS, the hemolytic moiety is to be purified and used to serologically assess host response to this toxin in acute and chronic streptococcal disease such as glomerulonephritis. Enzymatic methods to degrade Group B streptococci are being developed to better study its toxins [hemolysin(s) apparently different than SLS]. Various toxins, such as erythrogenic toxin, and cell components such as lipoteichoic acid, are being compared between Groups A, B, C, and G streptococci to determine potential relationships in disease production. The cell walls of Group A streptococci are being studied in the production of arthritis to study the genetic determinants of host responses.

Project Description:

Streptolysin S is the most powerful membranolytic agent known. Yet the role of this hemolysin of Group A streptococci in pathogenesis is unknown because no antibody response has been detected in either man or test animal. The toxin is made as an inactive precursor intracellularly--the majority associated with the membranous fraction of the cell. It has been found in high titer in all nephritogenic strains tested. The active hemolysin is likely a very small peptide which is very cationic and has high affinity for lipids. The objective is to purify the hemolytic moiety using the different chemical characteristics of the precursor and active hemolysin, to characterize it, and to determine its potential role in streptococcal disease such as glomerulonephritis. This and other toxins and inflammatory factors from streptococci are being characterized to determine potential roles in disease production.

(1) Streptolysin S

SLS precursor (measured in the presence of a carrier, RNA-core) was characterized in 1974-1975 in multiple types of Group A streptococci from both nephritogenic and non-nephritogenic strains and found to be in highest titer in the membranes and of highest specific activity in mesosomes of nephritogenic strains. On resuming this work in 1978, it was initially not possible to obtain these same high titers and strain differences from most of the previously lyophilized strains. The effect of different lots of media, additives (sugars and ions), buffers, cell fractionation steps, and even water source were studied to determine the reason for the difference, since fractions frozen in 1975 still showed the same high titers as when measured at that time. It was found that some strains had lost their high titer cellular SLS production for reasons that are yet uncertain. In other strains, the previously obtained high titers could be obtained by using muralysin (to degrade the cell wall to protoplast the streptococci) prepared in a slightly different way than before. Using new materials, the highest percentage of hemolysin precursor was again found in the membranes and the highest specific activity in the mesosomes of various types of streptococci. The absence of any hemolysin precursor in walls has also been confirmed. All precursor in the periplasmic fraction is sedimentable and probably reflects that associated with membrane fragments. A significant percentage of the precursor is also found in the cytoplasmic fraction and is sedimentable under appropriate conditions. Part, at least, appears to be ribosomally associated and may be a ribonucleoprotein since 75% of the activity precipitates from solution when treated with ribonuclease. The precise characterization of this cytoplasmic precursor is continuing.

That the precursor can be activated by vortexing with glass beads in the presence of RNA-core was previously reported. Studies of the chemical preparation and physical properties of the beads has shown the importance of small bead size (10-40 microns) and enhancement of bead utility by prewashing with water, by increasing % acetic acid to 60%, and by phosphate buffer but not by other acids or base. In some preparations of precursor, streptococcal RNA probably enhances the final titer. Numerous different detergents (nonionic, anionic, zwitterionic) have been tested but none alone activates the hemolysin precursor and those that solubilize the membrane inactivate the precursor. However, when precursor is vortexed with glass beads in the presence of a nonionic detergent such as Nonidet P40, active hemolysin is obtained in the absence of RNA-core. The activity of this hemolysin is very labile but can be stabilized by RNA-core. Work is in progress to further characterize this hemolysin in terms of its usefulness in a scheme to purify SLS. How the precursor is activated in vivo is uncertain, but studies are in progress to further investigate this. Since proteinases activate certain extracellular enzymes, the effect of streptococcal proteinase (obtained from Dr. Darrell Liu, BOB) was studied. Interestingly, although it had no effect to activate or degrade the precursor, it inactivated the active hemolysin suggesting a possible regulatory role of the proteinase on production of the extracellular hemolysin. The effects of other types of enzymes such as phospholipases on precursor activation remain to be determined.

The future course of this work will be to purify and characterize the hemolytic moiety, utilizing the differing characteristics of the precursor and active hemolysin. After purification of the precursor, it will be activated in the presence of a lipid carrier, purified by various means including chromatography in organic solvents, and then transferred to a synthetic nucleotide carrier bound to a thiopropyl Sepharose affinity chromatography column. After elution and further purfication by gel filtration and ethanol-acid treatment, the peptide and amino acid content will be determined.

(2) Toxins of Groups B, C, and G Streptococci

The development of a method to prepare protoplasts of Group B streptococci was needed to study various cellular toxins (such as hemolysins), since no suitable or efficient method had been previously described. Examination of some 50 to 100 different strains of streptococci of all groups and their phages for a phage-associated muralysin was unsuccessful. However, a Streptomyces globisporus enzyme, mutanolysin, supplied by Japanese investigators, degraded walls of all 5 Group B types as well as walls of Groups A, C, G, and H. Preliminary studies suggest that protoplasts of Group B can be prepared using this

enzyme. Interestingly, it was found by electron microscopy that when the enzyme was added in excess to cells of the different groups in hypotonic buffer, the gross cell form remained intact and leakage of cytoplasmic contents occurred from one area of the cell. Why the cell was not totally disrupted and whether only a part of the wall (instead of all) was degraded is uncertain, but under further study.

The presence of potential pathogenic factors in Groups A, B, C, and G is being investigated. Streptolysin S precursor has been found in Groups A, C, and G streptococci, but not Group B. Whether or not there are differences in cellular location among Groups A, C, and G has not yet been evaluated. Erythrogenic toxin production has been studied immunologically in Groups A, C, and G, but has been found thus far only in Group A. Lipoteichoic acid (LTA), suggested as important in streptococcal adherence, was found in all four groups in approximately equal amounts. Studies to determine the cell surface distribution of LTA are in progress.

(3) Peptidoglycan Induced Arthritis

The cell walls, or more specifically, the peptidoglycan, of streptococci, cause a severe arthritis in certain strains of rats. Although much is known about the effects of enzymatically modifying the peptidoglycan on its pathogenic potential, little is known about the determinants of the host in terms of whether arthritis will be produced. Using Group A streptococcal cell walls with various enzymatic and chemical modification, arthritis production is being evaluated in outbred and inbred strains of rats to determine what genetic factors are responsible, how the peptidoglycan is transported to the joints, how the inflammatory response is generated there, what specific bacterial components modulate this process, and how the process can be stopped by treatment of the host. (In collaboration with Dr. R. Wilder)

Publications:

None

LABORATORY OF VIRAL DISEASES
1979 Annual Report
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PHS-NIH
SUMMARY REPORT

ANNUAL REPORT OF THE LABORATORY OF VIRAL DISEASES, NIAID
October 1, 1978 - September 30, 1979

Dr. Wallace P. Rowe
Chief, Laboratory of Viral Diseases

There was a major administrative reorganization of LVD during the past year, consisting chiefly of removal of the LVD units located in Building 5. The units of Drs. Mattern, Takemoto, Lewis, and Jaouni have been reassigned. In effect Dr. Levy's unit has also been reassigned, but the formal transfer has not yet taken place. This reorganization has the salutary effect of consolidating LVD into a more coherent group both spatially and programmatically, and of greatly simplifying administrative responsibilities for the Laboratory Chief. The laboratory now consists of the unit of Drs. Rowe and Hartley plus that of Dr. Hoggan.

In addition to the change in the lab structure, there is a significant change in program. Both Drs. Rowe and Hoggan are now involved in generation of a new program involving molecular cloning of C-type viral genomes by recombinant DNA techniques. This program is under the general direction of Dr. Malcolm Martin, and involves collaboration with workers in LVD, NCI, Johns Hopkins, and MIT. This work is in part an outgrowth of the recombinant DNA risk assessment experiments of Drs. Martin and Rowe and will involve both risk assessment and basic scientific studies.

The involvement of the laboratory chief in the scientific and policy making aspects of recombinant DNA research again occupied a major portion of his time during the past year, but his term on the NIH Recombinant Advisory Committee has now expired and hopefully the time spent in this activity will decrease drastically.

Staff changes in LVD during the past year included the arrival of a Research Associate, Dr. Susan Light, and the return of Charles Buckler to work in LVD following virtual completion of his Ph.D. program at Johns Hopkins.

Dr. Rowe received a major award, the Paul Ehrlich-Ludwig Darmstaedter prize, which was shared with Drs. Graffi and Mühlbock. The prize was presented by President Scheel of The Federal Republic of Germany at a ceremony in Frankfurt.

Some of the highlights of the scientific accomplishments of the past year follow.

Additional MuLV chromosomal loci mapped. Seven ecotropic virus-inducing loci have now been precisely located on mouse chromosomal DNA. In addition to the five previously reported, Akv-2 has been mapped to chromosome 16 (as determined by somatic cell hybrid analysis) and one of the C58 loci has been identified on chromosome 8. A fifth xenotropic virus inducing locus, that of C57L, has been found to be at the same site on chromosome 1 as the xenotropic loci in other strains previously studied. (Kozak, Rowe)

Virus-inducing chromosomal locus identified by restriction blotting techniques. The Akv-1 locus can be specifically detected in cellular DNA by restriction enzyme analysis using the Southern blot technique. Using this technique it will eventually be possible to analyze viral chromosome loci in various mouse strains with great precission. (Steffen, Weinberg, Rowe)

Correlates of lymphomagenicity identified. Certain MCF viruses, which represent genetic recombinants of ecotropic and xenotropic MuLV, markedly accelerate the appearance of lymphoma in AKR mice while others are inactive. Comparisons of biological and biochemical characteristics of leukemogenic and non-leukemogenic isolates indicate that the former are isolated from thymic tissue or neoplasms of thymic origin, replicate efficiently in AKR thymus, are relatively restricted in ability to infect NFS mouse embryo cells as compared to SC-1 cells, and have in common certain characteristics of their RNA oligonucleotide fingerprints. Non-leukemogenic isolates are non-thymic in origin, unable to replicate in thymus, show the reverse pattern of infection of NFS and SC-1 cells, and have closely similar RNA fingerprint patterns which are clearly distinct from those of leukemogenic isolates. Recognition of these differences should assist in identifying the portion of viral genome controlling thymotropism. (Cloyd, Hartley, Rowe; Hopkins, MIT)

Genetic control of sensitivity to infection by MCF viruses under study. Ability of the virus to replicate in a given host is a necessary event in MCF virus induced lymphomagenesis, and evidence of genetic control of sensitivity to infection has been obtained both *in vivo* and *in vitro*. In addition to Fv-1, several other genes are clearly involved in determining susceptibility. (Hartley, Cloyd)

Identification of xenotropic viruses with MCF-related sequences. Although the ecotropic parentage of MCF viruses can be clearly identified as the endogenous ecotropic virus of a given mouse strain, the putative xenotropic parent is unknown. Certain xenotropic viruses but not others have been found to contain genome sequences that complement ecotropic viral DNA probes in saturating the entire genome of an AKR MCF virus. The significance of this finding in precisely identifying the second partner in the origin of an MCF recombinant is not yet clear, but these studies confirm involvement of xenotropic virus in the genesis of recombinant MuLV. (Chattophadhyay, Hartley, Rowe)

Protein sequence homology among AAV proteins. Tryptic and chymotryptic maps of the adeno-associated virus (AAV) structural proteins show extensive areas of sequence homology further suggesting that all three arise from a common precursor. (Lubeck, Lee, Hoggan, Johnson)

Appearance of extrachromosomal viral DNA in carrier culture. Long term passage (40-80 cell transfers) of AAV integrated human cell clones show small amounts of extrachromosomal viral DNA while earlier transfers show only integrated viral DNA. (Chan, Berns, Hoggan, Houseworth)

Relatedness of human parvovirus to rat virus. Three human cell specific parvovirus isolates (Kirk, HS-3 and PVZgd) demonstrate strong one way immunologic cross reactivity with the rodent rat virus (RV) and each possess the same three structural polypeptides. (Hoggan, Sears)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00011-14 LVD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Studies of Small DNA Containing Viruses Belonging to the Family Parvoviridae.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: M. D. Hoggan Staff Scientist, LVD, NIAID

COOPERATING UNITS (if any)
K. I. Berns, Univ. of Florida College of Medicine, Gainesville, Florida.
F. Brent Johnson, Dept. of Microbiology, Brigham Young Univ., Provo, Utah.

LAB/BRANCH
Laboratory of Viral Diseases

SECTION
Viral Oncology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	1	2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Ongoing studies of parvovirus have shown that long passage of integrated AAV carrier cell clones may show small amounts of extrachromosomal viral DNA. Studies of the polypeptides of "human" parvoviruses demonstrate that these viruses are structurally and immunologically closely related to rodent (RV) parvovirus and that these viruses undergo a post assembly maturation process involving protolytic cleavage. It has been further shown that the immunogenic expression of a cross reacting antigen between large plaque rat virus (LPRV) and minute virus of mice is linked to the host cells in which these viruses are grown. Continued studies of AAV proteins have shown that each of the three recognized structural polypeptides are closely related and show a great amount of protein sequence homology. One unexpected finding is that all of the polypeptides of canine AAV (CAAV) appear 10-20% larger than those of all other AAV's thus far studied. We have some evidence that suggests this is the result of a block in CAAV virion maturation.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To use molecular biological as well as classic physical-chemical, immunological and epidemiological techniques to elucidate the mechanisms of virus replication, persistence and genetic evolution of viruses belonging to the Family Parvoviridae for a better understanding of their natural history and role in disease.

Major Findings

Appearance of extrachromosomal viral DNA after prolonged culture of integrated AAV carrier clones: Soon after initiation of AAV carrier clones, multiple copies of the virus genome can be detected in host cell DNA with no detectable virions or extrachromosomal viral DNA. However, after prolonged cultivation (40-80 cell passages) small amounts of unintegrated viral DNA appear in the cell cytoplasm and can be detected using the Southern blotting technique. These results explain why virus is much easier to induce with helper virus in some high passage clones.

Polypeptides of "human" parvovirus strains Kirk, HS-3, and PVZgd indistinguishable from those of rodent parvovirus (RV): Kirk, HS-3, and PVZgd viruses replicate only in human cells yet they show strong one way serological reactivity with sera produced against various strains of RV which replicate to high titer only in rodent cells. Comparative studies of the proteins from these "human" and "rodent" viruses showed them to be indistinguishable from each other although they each could be easily distinguished from other members of the genus parvovirus. Further studies showed that, as with other members of this genus, the major polypeptide on the virus capsid at the time of assembly is a middle sized protein of about 70-72,000 MW. Later during the course of the infection, during maturation, this protein is proteolytically trimmed to a slightly smaller protein about 68-70,000 MW. Both the latter are more stable in CsCl and demonstrate a higher efficiency of infection.

Immunogenic expression of a cross reacting antigen between large plaque variant of rat virus (LPRV) and minute virus of mice (MVM) linked to host cells: We have carried out detailed studies of the LPRV variant which show that this variant exhibits striking differences from wild type RV in virulence in the experimental animal as well as in the size of plaque produced in tissue culture. The number of plaque forming units (PFU) required for killing newborn hamsters is 50 to 1000 times greater for LPRV than it is for wild type RV even though it produces plaques which are 3-20 times larger in rat nephroma cells. For these studies we have relied heavily on a cross reacting antigen between LPRV and MVM which is not normally expressed by small plaque (wild type) rat virus (SPRV). This cross reacting antigen is expressed on the surface of the virus and can be measured using hemagglutination inhibition, immune electron microscopy and serum neutralization and is coded for by the virus. However we find that only hyperimmunized animals injected with purified MVM prepared from virus produced in rat cells develop the cross reacting antibodies to LPRV. On the other hand, all sera from animals hyperimmunized with purified LPRV grown in rat nephroma cells react with all strains of MVM

while no sera from SPRV immunized animals show cross reacting antibody. Using polyacrylamide gel analysis the virion associated polypeptides from LPRV and SPRV cannot be distinguished but both are easily separable from MVM polypeptides.

Adeno-associated virus (AAV) structural protein sequence homology: AAV structural proteins have been purified and examined for areas of sequence homology between each of the 3 virion associated polypeptides. Tryptic and chymotryptic maps demonstrate extensive areas of homology and have a similar amino acid composition. Although we showed earlier that each polypeptide could be distinguished immunologically using FA antigen induction, there is a large amount of antigenic identity when using the more sensitive radio-immunoprecipitation assay. With one exception, all peptides contained in the smallest polypeptide are found in the middle and largest size peptides. These data further suggest that either a large precursor (120,000 MW) protein is cut with overlapping segments or the largest polypeptide is itself modified by some proteolytic action into each of the two smaller units. Tertiary structure on the virion is also important in specificity since various AAV show a high degree of specificity when antibody is raised against whole virions while much crossing occurs when antibody is raised against dissociated virus.

Canine adeno-associated virus (CAAV) exhibits uniquely large constituent polypeptides when compared to other members of the same genus: As has already been noted, various types of primate, bovine, avian, and canine AAV's show little or no serological cross reactivity when tested using sera produced against whole virions, even though their genomes share between 60-80% homology. On the other hand studies utilizing sera produced against capsid polypeptides all show cross reactivity. Our recent studies using ^{125}I exogenously labelled polypeptides from a number of different AAV types showed very little differences in MW between the polypeptides of the various AAV with the exception that CAAV polypeptides were 10-20% larger. The CAAV polypeptides can be easily distinguished from the others when coelectrophoresed on the same gel. It is unlikely that this represents an increase in coding capacity since the genomes of all AAV's are quite similar in size rather, it probably represents a block in some maturation step. We have previously shown that 2 populations of AAV capsids, each containing a complete viral genome, are produced during an adenovirus-helped lytic infection; these can be separated based on their relative specific density in CsCl. We have further shown that the more dense population can be immunologically distinguished from the mature population, is less stable and shows a reduced infection efficiency. These results and the results on the maturation process of members of the genus parvovirus taken in concert that AAV maturation may indeed involve a similar post assembly proteolytic cleavage. Although normally this process may not be seen because of rapid turnover, for some yet to be determined reason it is blocked in CAAV. Other data which supports this theory is the fact that we have never seen a major CAAV band in the density range of 1.40 g/cc and the very small full band which is seen is in the density range of 1.45 g/cc. This may also account for the extremely low infectivity titers we find for this virus.

Significance to Biomedical Research and the Program of the Institute

The Institute has long supported a program on slow viruses, persistence and their role in disease. Parvoviruses not only provide simple (genomic size less than $2x10^6$ MW) virus models for elucidating basic mechanisms of virus/cell interaction and persistence, they are wide spread in nature and may hold the key to our understanding of gene expression. They may also provide answers regarding the etiology of as yet poorly understood slow progressive diseases such as amyotropic lateral sclerosis. We have in fact found that 1% of all human embryonic tissues tested contain the parvovirus AAV-2 in an integrated occult state. What is this virus genome doing? The recent finding that a "new" parvovirus has been isolated from a number of fatal epidemics of canine enteritis which is indistinguishable from the parvovirus known to cause panleukopenia of cats, is of great interest. Is it possible that the canine virus is in fact, a recently evolved variant of the feline virus? With man living so close to his pets is it possible for a similar mutant to evolve which infects him? Are the "human" parvoviruses which are so closely related to the rat virus actually variants of that virus? Could they become pathogenic for man? These questions are not only fascinating they could obviously be important to public health.

Proposed course

Because of the rapidly developing area of recombinant DNA research and the need to develop expertise in this area for the full exploitation of the problems in parvovirus research. Our section has devoted about 60% of our effort to learning these techniques during the last 3-4 months. We have done this in collaboration with Dr. W. P. Rowe and Dr. M. A. Martin using an ongoing problem on retroviruses. Since many of the problems in retrovirus research involve integrated sequences, host restrictions, etc., and are in many ways analogous to the problems of parvovirus carriage it is felt this temporary collaborative arrangement will result in great benefit to both projects.

Publications

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00012-17 LVD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Studies of Mouse Leukemia Viruses.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Dr. Wallace P. Rowe, LVD, NIAID
Dr. Janet W. Hartley, LVD, NIAID
Dr. Christine Kozak, LVD, NIAID
Dr. Miles W. Cloyd, LVD, NIAID (Untile August 1979)
Dr. Janet M. Ramseur, LVD, NIAID
Dr. Susan E. Light, LVD, NIAID (From July 1979)

COOPERATING UNITS (if any) S. Chattopadhyay, DCT, NCI; M. Martin, OSD, NIAID; H. Morse, T. Chused, LMI, NIAID; M. Potter, LCBGY, NCI; L. Old, E. Stockert, Memorial Sloan-Kettering Inst.; M. Collins, MBA; P. Pitha, S. Staal, Johns Hopkins Univ.; N. Hopkins, MIT; R. Weinberg, D. Steffen, MIT

LAB/BRANCH
Laboratory of Viral Diseases

SECTION
Viral Oncology Section

INSTITUTE AND LOCATION
National Institute of Allergy and Infectious Diseases, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
11.8	4.8	7.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The natural biology of the complex group of murine C-type RNA viruses is being studied using virological, genetic, and biochemical approaches. Continuing studies of the genetic transmission of both ecotropic and xenotropic virus classes have led to the chromosomal mapping of several additional virus-inducing loci. Studies of the recombinant MCF viruses in relation to murine leukemogenesis have delineated certain biological and biochemical correlates of the ability of a given MCF isolate to induce or accelerate leukemia, leading to the recognition of target tissue host range as the major determinant of leukemogenicity. Genetic control of sensitivity to MCF virus infection is under investigation in vitro and in vivo.

PHS-6040
(Rev. 10-76)

The major goal of this project is the analysis of the natural biology of C-type viruses of mice. In particular the genetic transmission of these viruses is investigated by the use of inbred, hybrid, and congenic mouse strains. Construction of such strains, chromosomal mapping by Mendelian and somatic cell genetic techniques, and analysis of the interplay of genes that regulate expression of the virus-inducing loci is a large program area. In addition, recombinant DNA techniques are now being employed to obtain molecular clones of viral genomes and of the endogenous genetic loci. Related to this project on leukemia viruses is the collaborative work on risk assessment of recombinant DNA research using tumor viruses cloned in E. coli as models of possibly hazardous post-vector systems.

Chromosomal mapping of endogenous MuLV genomes. We have continued our analysis of the chromosomal loci in various mouse strains that are responsible for induction of ecotropic and xenotropic MuLV. We have previously mapped the Akv-1 locus, as well as ecotropic loci in BALB/c, C57BL, B10.BR and C3H/Fg, and xenotropic loci in 4 inbred strains. During the past year we were successful in identifying the chromosomal location of two more ecotropic virus-inducing loci: Akv-2 (on chromosome 16 as determined by somatic cell hybrid analysis) and one of the loci of C58 (on chromosome 8 near but probably not allelic with the locus carried by C57BL). In addition, we have mapped a fifth xenotropic locus, of C57L, and have found that it is at the same chromosome 1 site as in the previously mapped strains.

A major objective over the next year will be to map similar loci occurring in a wild mouse population that is probably the progenitor of many laboratory mouse strains; that is the Kyushu mouse (Mus molossinus) which carries an ecotropic virus indistinguishable from that carried by AKR with respect to its restriction map. Another major goal of the next year will be to characterize the endogenous loci by restriction enzyme analysis using the Southern blot procedure. In collaborative studies with Drs. Weinberg and Steffen at MIT we have shown that the Akv-1 locus can be recognized in restriction blots as a distinct band in the gel that segregates with the inducibility of virus. Once radiolabelled probes corresponding to specific regions of the AKR viral genome are available from the molecular cloning studies, it will be possible to characterize all of the ecotropic endogenous virus-inducing loci by the same technique. The question of allelism of various ecotropic genomes can then be studied in precise detail.

A particularly strong feature of our program in this regard is the availability, through Dr. Kozak, of segregating somatic cell hybrids containing only one or two mouse chromosomes, thus making it possible for us to examine particular genomes biologically and biochemically, separately from loci on other mouse chromosomes.

A number of the chromosomal genomes are being bred into NFS Swiss mice for production of congenic mice carrying various ecotropic and xenotropic virus loci. With the increasing success of mapping these loci we have found that a number of them are closely linked to morphologic markers such as genes for coat color or skeletal abnormalities; we are now making a number of congenic

lines carrying the virus loci in coupling with the morphological markers. This greatly simplifies analysis of segregants. Also, breeding the morphological markers unlinked to virus-inducing loci into the mouse strain that carries such a locus at the closely linked site provides a highly efficient way of producing mice congenic for the absence of a virus-inducing locus.

An outgrowth of the multidisciplinary genetic studies has been further confirmation that the virus-inducing loci consist of integrated viral genomes. We have shown previously that the Akv-1 locus contains at least a portion of the MuLV genome, but it was not possible to say if it contained the entire set of sequences. The restriction blots from the Steffen-Weinberg study indicate that the viral sequences at Akv-1 generate the same internal fragments when cleaved with a multiple cut enzyme as does the viral genome obtained from cells exogenously infected with AKR virus. This provides strong evidence that the locus contains the viral genome in unpermuted orientation and without any major intervening sequences. Also, the somatic cell hybrid studies of Dr. Kozak have shown that a segregating hybrid cell line containing only chromosome 1 derived from BALB/c, which carries a xenotropic inducing locus on this chromosome, is inducible for xenotropic virus; thus the genetic information must be on that chromosome and is presumably at the locus shown previously to determine inducibility.

Biology of MCF viruses. The MCF (Mink cell focus-forming viruses), discovered in LVD several years ago, constitute a major area of study in spontaneous viral leukemogenesis. These viruses are genetic recombinants between ecotropic and xenotropic viruses that arise in somatic cells during the preleukemic period in a number of mouse strains.

The collaborative studies with Dr. Chattophadhyay have now identified several xenotropic viruses that contain genome sequences that complement the ecotropic viral probes in saturating the entire genome of AKR MCF viruses. Other xenotropic viruses do not contain this set of sequences. These studies are of importance in confirming that MCFs are indeed derived from xenotropic viruses and for indicating that there is significant heterogeneity between xenotropic virus strains with respect to this important set of sequences. Whether this means that some xenotropic viruses cannot become involved in generation of MCF viruses remains to be determined.

In the continuing studies of the natural history of the MCF viruses it is now apparent that they can occur in most mouse strains, that carry high levels of ecotropic virus, and that they are found in a high proportion of spontaneous hematopoietic neoplasms arising in mice that carry ecotropic MuLV. We have now established that the MCFs isolated from various mouse strains fall into two sharp categories. The strains obtained from thymic tissue or neoplasms or thymic origin are a distinct family as opposed to those derived from nonthymic neoplasms or from nonthymic lymphoid tissue of normal animals. The strains recovered from thymus are able to accelerate thymic lymphomagenesis in AKR mice, they replicate in the AKR thymus, they show a relative restriction of growth in NFS mouse embryo cells as compared to SC-1 cells, and as shown by our collaborator Dr. Hopkins of MIT they share several features of their RNA

oligonucleotide fingerprint. With one exception the nonthymic MCF strains show the opposite pattern with respect to these phenotypes, that is they are unable to replicate in AKR thymus or to accelerate AKR leukemia, they infect NFS cells more efficiently than SC-1 cells and they have a different pattern of RNA fingerprints. One exceptional strain is intermediate with respect to all of these properties. These findings are of particular interest in that they point out the importance of tissue host range as the major determinant of leukemogenicity and they allow us to examine several regions of the viral genomes as the possible determinant of thymotropism.

Among the nonthymotropic MCF strains, several are clearly able to infect of splenic cells; this property is not shared by the thymotropic strains. Attempts will be made to identify the target cells of this subclass of MCF viruses.

The nonthymotropic MCF viruses, although recovered in many cases from hematopoietic neoplasms, have been completely devoid of ability to induce leukemias. These viruses, having arisen by recombination between endogenous viruses, may not have been selected for ability to initiate infection from without in their target cells. We will attempt to induce leukemias with these strains by using phenotypic mixing as a mechanism for introducing these viruses into potential target cells.

Genetic studies of susceptibility to MCF virus infection. Studies have been carried out both in vivo and in vitro on the ability of cells of different mouse strains to be infected by MCF viruses.

The studies in vivo have been confined primarily to tests for leukemogenicity by various MCF isolates in a variety of mouse strains. The only strain that was consistently sensitive is AKR, in which thymotropic MCF strains consistently accelerated thymic lymphomagenesis. C3H/Bi, the strain that is maximally sensitive to Gross passage A virus, was partially susceptible to MCF-247, as were NFS mice carrying AKR virus-inducing loci and showing the high ecotropic virus expression phenotype. NFS mice without the virus-inducing loci were refractory to MCF-247 leukemogenesis as were C57BR and, surprisingly, the high virus strain C58. In crosses between AKR and NFS, susceptibility to MCF-247 was dominant; and in backcrosses to NFS half of the mice were sensitive. This 50% ratio did not signify a simple one gene Mendelian effect, however, since all of the mice developing lymphoma were from the segregants inheriting AKR ecotropic virus. This confirms the earlier observation with the NFS congenics that presence of ecotropic virus facilitates MCF lymphomagenesis. We infer that several other genes segregating in this cross must be involved in determining susceptibility to MCF leukemogenicity. H2 type was not associated with sensitivity nor was the ability to mount a humoral immune response to the 247 virus.

In crosses between AKR and the resistant C58 strain, resistance to lymphomagenesis was dominant and in backcrosses to AKR again a one gene Mendelian ratio was observed. In this case all of the segregants carried ecotropic virus. Preliminary data indicate that susceptibility correlates with inheritance of the Fv-1-Gpd-1 region from AKR. Further preliminary studies suggest that the subline of C58 employed in these studies may actually carry a unique resistance

allele at Fv-1.

In the in vitro studies of susceptibility of tissue cultures to infection by MCF-247 and other N- or NB-tropic MCF virus strains, the unexpected observation was made that DBA/2 and DBA/1 mice are 30- to 100-fold less sensitive to the infection than other Fv-1^n mice. This resistance does not segregate with Gpd-1 and hence must be due to a gene other than Fv-1. A possible association with xenotropic virus expression in these mice will be investigated.

Studies of tumorigenesis in mice by restriction fragments of polyoma viral DNA. In the course of the polyoma risk assessment studies carried out in collaboration with the Recombinant DNA Unit, OSD, the striking observation was made that polyoma DNA inactivated by restriction enzyme cleavage in the distal portion of the early region showed enhanced oncogenicity for hamsters. To further investigate this phenomenon, we have carried out pilot studies in mice using polyoma DNA cleaved with enzymes that prevent it from being infectious. This was considered a possibly interesting area of study since polyoma tumorigenesis in mice is accompanied by high levels of productively infected cells, and the tumors consequently produce infectious virus. The cleaved DNA preparations should permit study of non-infectious mouse tumors. To date, non-infectious DNA preparations have induced a small number of tumors including one osteoma, one sarcoma, and two endotheliomas. The sarcoma has been established in serial transplantation passage and may be of much use for analysing transplantation antigens.

Future Course of the Project. The major components of the program will continue along the same lines as in the past few years. In addition much effort will be devoted to the collaborative program on recombinant DNA technology as applied to the analyses of endogenous C-type viral genomes. It is expected that this area of study will develop rapidly and move into a wide variety of areas involving the genetic nature of endogenous viral loci, the role of recombination in generating viruses from them, the control mechanisms involved in regulating expression of ecotropic and xenotropic endogenous viral loci, further insights into the origin of MCF viruses, and the role of various genetic regions of the virus in determining thymotropism and lymphomagenicity.

Publications

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LABORATORY OF MICROBIAL STRUCTURE AND FUNCTION
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RESEARCH HIGHLIGHTS

The following sections provide brief descriptions of significant findings during the past year. Eighteen publications have appeared in print, seven have been accepted for publication, and several others are in preparation.

PERTUSSIS

One of the most promising findings has been the development of specific affinity chromatography columns for the purification of pertussigen. With the aid of these columns, highly purified preparations of pertussigen have been obtained. This method should allow us to fully characterize this substance in the near future. Another important contribution was the development of methods to purify and crystallize the fimbrial hemagglutinin (FHA) from Bordetella pertussis. It was shown, contrary to what has recently been published, that when FHA is totally free of pertussigen, it does not protect mice from experimental pertussis, while highly purified pertussigen does. (Munoz, Arai, Sone)

It has been established that pertussigen, agglutinogen factor 1, and fimbrial hemagglutinin are three distinct substances. (Munoz, Arai)

One of the many biological properties of pertussigen is its ability to increase antibody production to antigens given with it. An unusual characteristic of this adjuvant action is that the IgE response is almost selectively increased. In our studies on the mechanism by which pertussigen increases the IgE response to hens' egg albumin, we have found that: 1) pertussigen acts differently than endotoxin or Concanavalin A, two substances that also can stimulate IgE production; 2) pertussigen given 3 days before antigen still acts as an adjuvant for IgE production; 3) pertussigen seems to act directly on lymphocytes probably by eliminating suppressor T cells; 4) anti-pertussigen serum, when given simultaneously with or 3 days after pertussigen, can suppress the adjuvant action of pertussigen; and 5) in spleen cells from mice that had received radioactivity-labeled preparations of pertussigen 3 days prior to removal of the spleens, a peptide of molecular weight 18,500 to 19,000 was demonstrated, indicating that pertussigen may remain in spleen cells for some time. (Sadowski)

We have had success in culturing the β-cells of the pancreas of neonatal mice. These cultures will be used to study the mechanisms by which pertussigen stimulates production of insulin. (Bergman)

MICROBIAL COMPONENTS IN CANCER IMMUNOTHERAPY

The goals of this project are to fractionate microorganisms, isolate those components that possess immunopotentiating ability, and to provide acceptable agents to clinicians for testing.

We have reported previously that a high incidence of regression of line-10 tumors was obtained with materials unrelated to tubercle bacilli provided they were combined with trehalose dimycolate (P3 or purified cord factor provided by Dr. R. Parker, Hamilton, MT, under NIAID contract). Particularly rapid destruction of tumors was observed when they were treated with endotoxic glycolipids (ReGl) from O antigen (polysaccharide)-deficient Re mutant strains of Enterobacteriaceae combined with P3 and up to 100% cures were obtained. Furthermore, ReGl synergistically enhanced the potency of CWS or CWS + P3 combinations. However surprisingly, endotoxic lipopolysaccharides (LPS) from all wild type strains so far tested failed to cause tumor regression. Other experimental evidence accumulated indicated that no correlation existed between endotoxic potency per se and tumor regressive potency. Therefore, from a practical point of view our interest centered upon the importance of the toxicity of ReGl. We explored chemical modification techniques hoping to selectively reduce the toxicity of endotoxic bacterial extracts and yet retain their ability to synergistically enhance the action of CWS. Whereas highly endotoxic LPS from wild type strains failed to cause tumor regression, we now report that acid hydrolysis of such LPS led to a residual fraction (RESI) that serologically cross-reacted with ReGl samples and provided high cure rates (90%) in the line-10 tumor model. RESI from Serratia marcescens was essentially nonpyrogenic for rabbits and 100 times less toxic for chick embryos than potent endotoxins. Both RESI and ReGl were found to contain peptidic substances; among the major amino acids were muramic acid, alanine, and glutamic acid. The amino acid content of ReGl was reduced by microparticulate silica gel chromatography, and this procedure provided an endotoxic fraction, B4 (prepared by Dr. R. Parker under NIAID contract), with reduced tumor-regressive activity. We considered that precursor or autolysis products of the peptidoglycan moiety of CWS may have been co-extracted with the endotoxic glycolipids. Indeed, antitumor activity could be restored to the endotoxic B4 by the addition of synthetic N-acetyl-muramyl-L-seryl-D-isoglutamine (MDP), which is considered the minimal structural unit responsible for the adjuvant action of the microbial cell wall. It is suggested that MDP may act as an adjuvant to an antigen which may have cross-reactive determinants associated with the endotoxin itself. Such antigens appear to be cryptic or sterically hindered from being effective in polysaccharide-rich LPS from wild type bacteria but are exposed in ReGl from polysaccharide-deficient mutant bacteria and RESI which was prepared from LPS by acid hydrolysis. Dr. Cantrell, in collaboration with Dr. Springer, found line-10 tumor cells, ReGl, and B4 to have components that cross-react with T antigen, the precursor of human blood group MN antigen. Studies by these workers are in progress to determine whether the cross-reactive antigens shared by line-10 tumor cells and microbial components play a role in tumor regression.

Whereas synthetic MDP appears to be a very effective adjuvant which can replace CWS, it was surprising to find that, in contrast to CWS, MDP greatly enhanced the susceptibility of guinea pigs to endotoxic shock. Results of our studies indicated that caution must be exercised in the application of MDP as a tumor therapeutic ingredient, or as an adjuvant in general, in combination with endotoxic products, such as Pseudomonas vaccine (Pseudogen) whose antitumor activity is presently being evaluated by others in human patients. (Ribi, McLaughlin, Cantrell, Schwartzman)

MECHANISMS OF IMMUNOPOTENTIATION BY MICROBIAL COMPONENTS

The primary aim of this project is to define, at the cellular, biochemical, and molecular levels, the mechanisms involved in immunopotentiation by microbial components. It was found that mycobacterial cell wall skeleton when combined with endotoxic extracts from Salmonella rough mutants (Re glycolipid) (ReGl) produced synergistic, not additive, tumor-regressive responses. N-acetyl-muramyl-L-alanyl-D-isoglutamine (adjuvant dipeptide) replaced cell wall skeleton in this synergy with endotoxic extracts. However, only upon treatment of adjuvant dipeptide with P3 was the adjuvant dipeptide combined with ReGl active against both the dermal and metastatic tumors. Without P3, regression occurred in dermal tumors only, with the treated animals succumbing to metastatic tumor growth. A covalently lipidated adjuvant dipeptide (provided by Drs. I. Azuma and Y. Yamamura) when combined with endotoxic extracts was effective against both dermal and metastatic tumors. These studies identified critical structural components of the mycobacterial cell wall responsible for the synergistic antitumor activity observed upon combination of cell wall skeleton with ReGl. It is thought that the heightened efficacy of either covalently or noncovalently lipidated adjuvant dipeptide reflects a critical mechanism of either a prolonged depot effect at the site of injection or a preferential processing of the lipidated adjuvant dipeptide by immune cells. (McLaughlin, Ribi)

As an extension of these findings, a collaborative study with Dr. G. Jones, Syntex Corporation, was initiated to investigate the use of various synthetic analogs of adjuvant dipeptide in treatment of tumors. Certain, extremely potent, antitumor preparations were discovered; all active ones required combination with P3 in an oil-in-water emulsion for expression of antitumor activity. (McLaughlin) In collaboration with Dr. R. Toubiana, France, synthetic analogs of trehalose dimycolate, such as trehalose desoxypalmitopalmitate, were found to act in concert either with endotoxic extracts of synthetic muramyl dipeptides to produce significant incidences of tumor regression. Thus, we have successfully simulated some of the biological activity of mycobacterial components through the use of synthetic substitutes. The use of radiolabeled muramyl dipeptide will be extremely important in mechanistic studies planned. Some success in synthesis of radiolabeled material for such studies has been made, and additional work is in progress. (Schwartzman) These synthetic substances will greatly simplify the task of visualizing the biochemical steps leading to the phenomenon now called adjuvant activity or tumor-regressive activity.

IMMUNOLOGICAL ACTIVITY OF EUKARYOTIC AND PROKARYOTIC CELLULAR COMPONENTS

The administration of Corynebacterium parvum has been shown to have diverse effects on the immune system. Although C. parvum has been shown to act as an immunoadjuvant increasing cell-mediated and humoral immunity, it has also been demonstrated to suppress cellular immune functions.

Through collaborative efforts with investigators from several institutes, we have gained valuable information on the immunological activity of C. parvum.

Several observations during the past year have strongly suggested that the component(s) of C. parvum responsible for antitumor activity can be isolated and differentiated from the component that induces suppressor cell activation and inhibition of humoral and cellular immunity. Since our supply of strain-2 guinea pigs was reduced the past year, the biological and antitumor properties of C. parvum fractions were evaluated in mouse-tumor models. Fractions prepared by phenol-water extraction of whole cells were tested for lymphoreticular stimulation by measuring the degree of splenomegaly and blastogenesis. Using three syngeneically transplanted murine tumors, antitumor activity was evaluated by monitoring the rate of tumor growth in animals given the fractions admixed with tumor cells. Antitumor activity and lymphoreticular stimulation were associated only with the phenol-insoluble residue. The kinetics of these paralleled that observed with intact whole organisms. Neither the aqueous-soluble nor the phenol-soluble extracts had activity with respect to these criteria. Further inquiries were made into the nature of the active component in the residue fraction. Following solvent, protease, or nuclease treatments of the residue, high antitumor and splenomegaly-inducing properties were retained. However, after the carbohydrate moieties of the active residue were inactivated, these properties were significantly reduced. These results suggest that the active component is, in part, carbohydrate in nature and not free lipids, proteins, or nucleic acids. Conceivably, this substance is associated with cell walls since purified cell walls were active, but the degree of activity was less than that observed with whole cells or the residue.

We also investigated the immunosuppressive properties of the C. parvum fractions obtained by phenol-water extraction. Significant inhibition of primary and memory cytotoxicity against alloantigens was observed in animals given the residue fraction. In addition, this fraction stimulated the generation of suppressor cell activity capable of inhibiting the expression of memory cytotoxicity. On the other hand, the protein-rich phenol-soluble fraction inhibited humoral immunity as well as primary and memory cytotoxicity, but did not enhance suppressor cell activity. The aqueous-soluble fraction was inactive as measured by these parameters. Collectively, these findings clearly demonstrate that materials isolated from C. parvum differentially effect various aspects of the immune response.

Attempts were also made this year to isolate, in soluble form, the antitumor component of C. parvum. We found that the pyridine-soluble extract, when combined with adjuvant active muramyl dipeptide (MDP) and trehalose dimycolate (P3), completely regressed line-10 tumors in syngeneic strain-2 guinea pigs. The rate of regression was superior to that observed with either whole cells or phenol-insoluble residue. In addition, the pyridine-soluble

extract did not induce splenomegaly or hepatomegaly, whereas the pyridine-insoluble residue significantly increased these parameters. This is the first report of a soluble component isolated from C. parvum having increased antitumor activity when compared to whole organisms. Studies also demonstrated that inhibition of primary immunity was associated with the pyridine-insoluble residue and not with the extract having antitumor activity. Further testing is needed to determine whether these fractions stimulate suppressor cell activation. In addition, we plan to fractionate the pyridine-soluble extract with hopes to isolate a more homogeneous agent.

In further studies on the efficacy of specific immunostimulants in tumor regression, attempts were made to isolate tumor-associated antigens from both murine tumors and guinea pig ascites fluid. Solubilized tumor antigens prepared by KCl extraction of murine tumor cells induced high cure rates in animals given combination chemoimmunotherapy. Successful therapy was dependent on emulsifying the antigen in minute oil droplets. In addition, animals cured by specific immunotherapy had detectable tumor specific immunity as measured by antibody cytotoxicity and reaction of a challenge of tumor cells. Isolated tumor antigens from the ascites fluid of tumor-bearing guinea pigs, when combined with MDP and P3 and emulsified in oil droplets, were also efficacious in regressing tumors. (Cantrell)

STRUCTURE AND BIOLOGICAL ACTIVITY OF ENDOTOXINS

Several novel approaches were used to describe further the intricate relationship between the structure and function of bacterial endotoxin. Also, special efforts were made towards understanding the capacity of endotoxic materials to interact with and stimulate defined populations of lymphoid cells.

One major advancement was the extraction of endotoxic materials from cell walls rather than from whole cells of Re mutant gram-negative bacteria. These cell walls were treated with EDTA prior to the extraction of endotoxin and the resultant endotoxic material was soluble in both aqueous and organic solvents. Following fractionation by chromatography on microparticulate silica gel columns, some characteristics of the starting material (i.e., pyrogenicity for rabbits, mitogenicity and polyclonal B cell activation for endotoxin responder mice, and antigenicity) were retained by the purification products whereas other activities (i.e., toxicity for chicks and lymphocyte stimulation for endotoxin nonresponder mice) were diminished or lost. These results suggested that the starting endotoxic glycolipid may have contained at least two components with different biological activity.

Other studies analyzed the chemistry and biological activity of endotoxins extracted from pathogenic or nonpathogenic strains of Yersinia enterocolitica. Potent endotoxins were isolated and characterized from both strains of this organism which has been gaining importance in certain enteric and systemic infections.

To assess the effects of endotoxin on the in vivo induction and modulation of thymus derived lymphocytes (T cells), an antigen-specific proliferation assay system was used. Important findings using this system included: 1) the existence of a population of T cells which specifically recognized and reacted to antigens associated with endotoxin, 2) that endotoxin was a potent adjuvant in the in vivo generation of T cells specific for protein antigens, and 3) endotoxin-reactive T cells and the adjuvant effects of endotoxin were absent in genetic nonresponder mice. These important studies were relevant to the following points: 1) The discovery of endotoxin-specific T cells provided the potential that such T cells participate in immune responses to endotoxin. Therefore, we would like to recommend a re-evaluation of the paradigmatic idea that endotoxin is purely a "T cell independent" antigen. 2) The capacity of endotoxin to potentiate T cell responses to other antigens may provide, in part, an explanation for the immunotherapeutic activities of endotoxin described in this and accompanying progress reports.

Finally, bacterial endotoxins were tested for the capacity to inhibit tumor growth in endotoxin responder and nonresponder strains of mice. Admixture of endotoxin with viable tumor cells dramatically reduced the incidence of tumors in responder mice and had little or no effect in nonresponder mice. Results of other experiments suggested that the antitumor effectiveness of endotoxin, in genetic responder mice, depended upon elicitation of a host-mediated response, possibly involving T cells. (Ribi, Von Eschen, McLaughlin)

ANTIGENS AND CLASSIFICATION OF RICKETTSIAE

The latest results from a study of the variation in properties of eight strains of spotted fever group rickettsiae reinforce an earlier preliminary conclusion that there is considerable heterogeneity in properties of these rickettsiae in nature. These strains varied in their ability to cause fever in guinea pigs, the type of plaque produced in Vero cell monolayers, and in their reaction patterns with rabbit antibodies raised against the rickettsiae. One cluster of similar properties was observed: the virulent rickettsiae produced large clear plaques and belonged to the same serotype. (Anacker)

Investigations of two types of serologic tests for diagnosis of Rocky Mountain spotted fever indicated some promise of usefulness for these procedures. One test, a hemagglutination test employing human group "O" erythrocytes sensitized with an alkali digest of gradient-purified Rickettsia rickettsii, detected spotted fever antibody in patients as early as 3 days and as late as 3 years after onset of symptoms. The second test (directed by Dr. Hechemy of the New York Department of Health), the latex agglutination test, thus far has successfully demonstrated spotted fever antibodies in most patients tested. The latter test is extremely rapid, simple, and economical. (Anacker, Philip)

BIOCHEMICAL AND GENETICAL MECHANISMS OF OBLIGATE INTRACELLULAR PARASITISM

The primary objective of this project is the elucidation of the metabolic cooperation between parasite and host. Specifically, we are investigating the biochemical parameters involved in rickettsial parasitism of eukaryotes with the ultimate aim of determining the growth factors. The underlying genetic determinants involved in the expression of bacterial functions are being investigated by classical methods. Biochemical parameters mediated by plasmids, episomes and bacteriophage are an active part of this program. We have identified molecular interactions at the cell surface which can be considered as primary determinants of metabolic cooperation between host and parasite. Obligatory features of rickettsial functions have been discovered in purine nucleotide metabolism. Rickettsia typhi utilize host purine nucleotides directly from host cytoplasm via a mechanism which requires the oxidation of glutamate. These experiments have shown that unusual membrane functions contribute to the success of the parasite as a scavenger of critical host components. R. typhi conserves the purine nucleotide pool as adenosine 5'-monophosphate (AMP), whereas autonomously growing bacteria degrade AMP to smaller compounds.

Metabolic comparisons between epicellular (autonomously growing bacteria) and rickettsiae have revealed exploitable differences in antibiotic sensitivities. The cell wall directed antibiotic, fosfomycin, which enters cells via a specialized transport process inhibits the growth of R. rickettsii but not Coxiella burnetii or R. typhi. More importantly, this antibiotic restricts the growth of Rochalimaea quintana and Legionella pneumophila at an ED_{50} of 5 and 12 µg per ml, respectively. The concentration of fosfomycin in the blood of patients has been reported to be as high as 33 µg/ml which is clearly adequate for the inhibition of these to epicellular bacteria. We are currently investigating the feasibility of this antibiotic as an effective chemotherapeutic for L. pneumophila infections in guinea pigs. (Williams, McCaul, Peacock)

STRUCTURAL AND FUNCTIONAL RELATIONSHIPS OF BACTERIAL ANTIGENS IN THE IMMUNE RESPONSE

The objectives of this project are to characterize the antigens of the rickettsiae and other bacteria which are pathogenic for humans. We have been analyzing the structure and functional aspects of the immunomodulation by the cell wall and soluble components. Potential candidates for subunit vaccines and tumor-regressive components have been identified. The present whole cell vaccine for C. burnetii induces severe local and systemic reactions. Our objective was the removal of the highly toxic factor(s) from whole cells via organic solvent extraction methods. We have discovered a particulate component which is nontoxic, provides protection against lethal challenge in mice and guinea pigs and regresses the line-10 tumors in strain-2 guinea pigs. This particulate material can be derived from whole cells or cell walls of C. burnetii. Future studies on the fractionation of cell walls should yield information on the nature of the toxic factor(s) and

eventually the preparation of a nontoxic subunit vaccine against Q fever. Studies are currently being conducted to determine both the type and intensity of the immune response to antigen. (Williams, Ribi, Cantrell, Peacock, McCaul)

Soluble components derived from mechanically disrupted C. burnetii have been identified as putative early diagnostic antigens. At least three soluble antigens have been identified. Antibodies to one of these is expressed only during infections, whereas the other two are expressed during immunization. Attempts are being made to purify the antigens and to characterize their immunostimulatory mechanisms. We should be able to prepare a soluble component that will be useful as a vaccine and early detection as well as distinguishing between recent infections and chronically infected individuals. (Williams, Kindmark, Peacock)

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ADMINISTRATIVE REPORT

The Laboratory of Microbial Structure and Function (LMSF) was established during the reporting year. It is comprised of three sections: Pertussis Section, Molecular Biology Section, and Rickettsial Diseases Section. LMSF utilizes sophisticated biophysical, biochemical, analytical and synthetic chemical techniques to isolate and identify components of bacteria and rickettsiae or synthetic analogues thereof which then are studied in biological test systems for their immunogenicity and toxicity or other hazardous properties.

Dr. Steven M. Schwartzman was appointed Senior Staff Fellow, Molecular Biology Section, to function in his synthetic organic chemistry specialty. Dr. Thomas F. McCaul, Visiting Fellow, was reassigned to assist Dr. Jimmy C. Williams, Rickettsial Diseases Section, in electron microscopic studies. Dr. Claes Kindmark, Sweden, spent six months as a guest worker collaborating with Dr. Williams in the study of soluble rickettsial antigens. During a one-month visit, Dr. Emilio Weiss, Naval Medical Research Institute, Bethesda, collaborated with Dr. Robert N. Philip and Mr. Marius Peacock (Epidemiology Branch) and with Dr. Williams in studies dealing with biochemical and physiological aspects of legionaires disease. Under Services Contracts, Dr. Robert W. Wheat, Duke University, spent three months collaborating with Drs. John L. Cantrell and Edgar Ribi (Molecular Biology Section) in the fractionation of Corynebacterium parvum cells and bacterial endotoxins, and Dr. Raoul Toubiana, Paris, worked with Dr. Charles A. McLaughlin (Molecular Biology Section) for three months on synthesis of mycolic acid ester analogues. Dr. Werner Brehmer, Robert Koch Institute, Berlin, spent six weeks as a guest worker collaborating with members of the Molecular Biology Section in studies on tumor regression with microbial components. Dr. Gary Calandra, LSD, NIAID, spent a month in LMSF to make use of tools and techniques available here for the isolation and structural identification of peptides. Dr. Jin-ichi Sasaki, Visiting Associate, completed electron microscopic studies on a large portion of his project and returned to Japan. He will continue to collaborate with members of the Molecular Biology Section from his laboratory at Hirosaki University.

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HONORS AND AWARDS

The following activities reflect the recognition afforded staff of the laboratory by their peers in the scientific community.

Awards:

Dr. J. J. Munoz received the NIH Director's Award for exemplary research on the immunobiology of components of Bordetella pertussis and his contributions toward the development of an improved vaccine against whooping cough.

Editorial Boards of Journals:

Dr. E. Ribi - Infection and Immunity

Drs. J. L. Cantrell, C. A. McLaughlin, J. J. Munoz, E. Ribi and J. C. Williams reviewed manuscripts submitted to them by Cancer Immunology and Immunotherapy, Journal of Immunology, Infection and Immunity, Journal of Infectious Diseases, Journal of the National Cancer Institute, Molecular Immunology, Ad hoc reviewer NIAID's committee, NID, on "RMSF in an Animal Model" and CRC Press, Inc. "Pyrimidine Metabolism in Bacteria."

Professional Posts:

Dr. C. McLaughlin - Elected member, American Association for Cancer Research.

Dr. J. Munoz - Trustee of Stella Duncan Memorial Fund for Allergy Research, University of Montana, Missoula; staff affiliate, Microbiology Department, University of Montana, Missoula; and chairman-elect of Immunology Section of American Society for Microbiology.

Dr. E. Ribi - Visiting Professor, University of Texas Cancer System, Texas Medical Center, Houston, and staff affiliate, Microbiology Department, University of Montana, Missoula.

Invited Lectures and Participation in Meetings and Symposia:

Drs. R. K. Bergman, C. A. McLaughlin, J. J. Munoz, and E. Ribi participated in internationally sponsored meetings and symposia.

Honors and Awards (cont'd)

As in previous years, many staff were invited to present lectures at universities, most of which were done in connection with travel to annual meetings of national organizations. Three staff members presented invited lectures at five different colleges and universities.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00065-06 LMSF-EB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Antigens and Classification of the Rickettsiae

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. L. Anacker	Res. Microbiologist	LMSF NIAID
	R. N. Philip	Medical Director	EB NIAID
OTHER:	W. Burgdorfer	Res. Entomologist (Med.)	EB NIAID
	E. A. Casper	Nurse Director	EB NIAID
	T. F. McCaul	NIH Visiting Fellow	LMSF NIAID
	L. A. Thomas	Res. Microbiologist	EB NIAID

COOPERATING UNITS (if any)
Dr. Robert Lane, California Department of Health, Berkeley, CA

LAB/BRANCH Laboratory of Microbial Structure and Function and Epidemiology Branch, Hamilton, MT 59840

SECTION
Rickettsial Diseases Section and Epidemiology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.6	2.7	2.9

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long-range objectives of this project are to develop practicable procedures for classification of spotted fever- and typhus-group rickettsiae and to determine the nature and biological properties of rickettsial antigens and constituents. Current specific interests are: 1) serotyping rickettsiae by microimmunofluorescence, 2) relationship of rickettsiae by means of toxin neutralization and polyacrylamide gel electrophoresis assays, and 3) nature and properties of rickettsial antigens determined by other specialized techniques.

PHS-6040
(Rev. 10-76)

Project Description:

A variety of serological and physicochemical approaches is being utilized to classify rickettsiae within the spotted fever (SFG) and typhus groups and to isolate and characterize the antigens of these rickettsiae. Included among these procedures are microimmunofluorescence (micro-IF), polyacrylamide gel electrophoresis, and crossed immunoelectrophoresis (CIE).

(Anacker) With the completion of four sets of experiments, one phase of our study of the variations in properties of eight strains of SFG rickettsiae, isolated from several species of ticks, animals, or man, has been terminated. By ordinary light microscopy little morphologic difference was found among the various rickettsiae in stained smears of infected yolk sacs. Diameters consistently fell within the range of 0.3 to 0.4 μm and lengths averaged 0.7 to 1.1 μm.

On the basis of the other three tests, however, these rickettsiae were more easily differentiated. The rickettsiae were divided into three virulence groups according to the fever responses of guinea pigs inoculated intraperitoneally with 1000 plaque-forming units of rickettsiae. The strains of highest virulence induced temperatures greater than 39.6°C for 6 to 8 days. Strains of intermediate virulence caused only a few days of fever, whereas guinea pigs inoculated with strains in the third category were afebrile. Differences in areas under the fever curves for strains in the different virulence groups were significant at the 0.05 level.

Plaques produced by these selected strains on Vero cell monolayers served to distinguish most strains. Some strains produced large clear plaques, some large turbid or target-like plaques, and one strain a small turbid plaque. The ability of the rickettsiae to produce large clear plaques was correlated with virulence of these strains for guinea pigs.

With the aid of microagglutination tests, employing antiserum from rabbits inoculated with several small doses of formalin-killed rickettsiae purified by sucrose density centrifugation in a zonal rotor, these strains were classified into five distinct serotypes. The four most virulent strains fell within one serotype; the other four, essentially avirulent strains were all serologically different.

The above tests, as well as those mentioned in the 1977-78 report, indicate considerable diversity in properties of SFG rickettsiae isolated in the U.S. It is unclear whether these differences are great enough to warrant elevation of some of these strains to species rank or whether these differences represent normal deviations from the standards established for classical strains of Rickettsia rickettsii. Perhaps results from our recently resumed study of the antigens of these rickettsiae will help to clarify this problem.

Preliminary results have been obtained from a comparison of extracts of several of the presumed more distantly related strains by CIE. It appears most antigens demonstrated by this procedure, particularly those which produce the densest precipitates, are shared by these strains. Probably, these common antigens are the "group" antigens defined by their ability to react with complement-fixing antibody in sera against all SFG agents. Type-specific antigens have not yet been unequivocally demonstrated for these strains.

(Philip) Eight isolates by cell culture obtained during the 1977 Bitterroot Valley survey of Dermacentor andersoni ticks (see 1977-78 report), two representing each of the four serologic types identified by micro-IF including R. rickettsii, R. montana, R. rhipicephali, and the 369-C serotype were examined for other distinguishing biologic characteristics including pathogenicity for chick embryos, guinea pigs, and cell culture; stability during serial passage in Vero cells; antibiotic sensitivity; cross-immunogenicity in guinea pigs; and growth dynamics in cell culture. Strains within serotypes were similar to each other, but the serotypes could be readily distinguished one from another on basis of several biologic markers in addition to micro-IF reactivity. Some observations follow.

Pathogenicity for chick embryos. Strains of R. rickettsii gave characteristic death patterns (4 to 5 days) after the first passage in embryonated eggs. R. montana strains were fully adapted after the third passage in eggs, killing embryos regularly on the fourth to fifth days. R. rhipicephali strains could be maintained by serial passage in embryonated eggs but killed irregularly even after the eighth passage. The 369-C strains could be maintained in embryonated eggs but were not lethal for chick embryos.

Pathogenicity for guinea pigs. R. rickettsii strains gave characteristic febrile responses beginning on the fourth day and accompanied by typical scrotal reactions and occasional deaths. R. montana and R. rhipicephali rarely gave rise to fever or scrotal reaction. 369-C strains were completely nonpathogenic for guinea pigs. The numbers of plaque-forming units per guinea pig infectious dose (determined by serologic response) were approximately 10^2 for cell culture-grown R. rickettsii strains and 10^5 for R. montana and R. rhipicephali organisms. The serotypes could be distinguished by the pattern of micro-IF antibody response.

Cytopathogenicity in cell culture. All serotypes were cytopathic for chick embryo cells. In Vero cells the serotypes could be distinguished on basis of plaque morphology, time of appearance of plaques and microscopic appearance of the cytopathic effect.

Stability after serial passage in cell culture. Each strain was examined by micro-IF for serologic change after 50 serial passages in Vero cells. All but one of the filial strains were indistinguishable from the parental strains. The exception was an instance where R. rickettsii

apparently "changed" to R. rhipicephali between the 40th and 48th passages. This is thought to have resulted from spurious contamination and overgrowth of R. rickettsii by R. rhipicephali during passage.

Antibiotic sensitivity. The strains were compared in Vero cell plaque-reduction tests for sensitivity to penicillin, streptomycin, neomycin, tetracycline, erythromycin, rifampin, and gentamycin. No major differences in the spectra of strain sensitivity were noted except to penicillin G. Ten times the dose of penicillin necessary to inhibit plaque formation by 369-C strains were required to inhibit plaque formation by other serotypes.

Cross-immunogenicity in guinea pigs. Some information on immunologic relationships among serotypes was obtained by the ability of guinea pigs previously inoculated with graded doses of the eight representative strains to withstand challenge by 1000 guinea pig ID_{50} of virulent R. rickettsii. The R. rickettsii strains conferred complete protection. R. montana and R. rhipicephali strains gave partial protection. The 369-C strains did not infect and did not protect against challenge.

Growth dynamics in cell culture. The growth dynamics in slide-chamber cultures of irradiated Vero cells were determined for the 369-C agents. Growth characteristics were similar to those of R. rickettsii except that intranuclear location is infrequent, an extremely large population of organisms can be supported by infected cells before cell death occurs and obversely, these organisms do not seem to have a mechanism for egress from infected cells. Transfer of organisms appeared to occur via intercellular bridges.

In 1977, isolates of rickettsiae representing three different serotypes (R. rickettsii, R. rhipicephali, and R. montana) were obtained from 18 ticks collected in the vicinity of Como Lake in an area less than one kilometer square. In 1978, this area was systematically flagged during spring and summer and the collected ticks were examined for rickettsiae. The serotypes were determined both by sequential FA-staining of the hemolymph and isolation of organisms in Vero cell culture. Three hundred seventy-one ticks were collected during the season. Eleven were positive by hemolymph test for SFG rickettsiae. Isolates were obtained from nine, eight of which were R. rhipicephali and one is as yet unclassified. Thus, no strains of R. rickettsii or R. montana were recovered in a locus which readily yielded isolates of these rickettsiae during the previous year. Two strains of R. rhipicephali were established in laboratory tick lines and carried through one complete generation. R. rhipicephali was also identified in a third female tick. Six normal nymphs that were fed on the same animal with the infected female acquired R. rhipicephali infection. However, F_1 larvae and nymphs were infected with organisms identified as 369-C, and F_1 adults were no longer infected with rickettsiae. Thus, it appeared that either the original female was dually infected with two serotypes of rickettsiae, or antigenic variation among the infecting organisms occurred during or after transovarial passage.

The former alternative appears to be the most likely possibility in view of the fact that the guinea pig on which the parental female fed responded with both R. rhipicephali and 369-C antibodies.

This year, in collaboration with the California State Department of Health (Lane) we are screening ticks obtained from coastal areas of California for presence of SFG rickettsiae and typing the isolates by immunofluorescence. The preponderance of ticks obtained by flagging were D. occidentalis from three areas: U. of California Hopland Field Station, north of San Francisco, U. of California Hastings Natural History Reservation, Monterey peninsula, and Torrey Pines State Park, San Diego. By hemolymph test, 165 (19%) of 875 ticks had rickettsia-like organisms. Thus far, 46 isolates of SFG rickettsiae have been recovered. Sequential staining by direct immunofluorescence indicated an antigenic relationship to R. rhipicephali by most strains recovered from D. occidentalis. One of these isolates was nonpathogenic for guinea pigs and embryonated eggs. One isolate from D. occidentalis was closely related to R. rickettsii in cross-micro-IF tests of mouse antisera. This strain gave scrotal reaction but no fever in guinea pigs and was pathogenic for chick embryos. An isolate from one of two D. variabilis collected serotyped as the 369-C agent. A fourth isolate from Ixodes pacificus appears to be different from all of the other isolates but has not yet been characterized. Thus, it appears that SFG agents are widely prevalent in ticks from coastal areas of California and on occasion certain of these organisms may play an etiologic role in the sporadic RMSF-like illnesses that occur in areas where the usual tick vectors of RMSF are not present.

In the future, we will continue our attempts to identify group- and type-specific antigens in extracts of various SFG rickettsiae with the aid of immunoelectrophoretic procedures. If the existence of a type-specific antigen for the virulent strains of R. rickettsii can be established, an effort will be made to prepare a type-specific antiserum. Conceivably, such a serum could be of considerable value in determining whether the rickettsial agents found in ticks feeding on humans have the potential to cause disease and thus, whether the individuals should be treated with antibiotics. Our second major effort will be the continuation of our attempts to isolate and identify the protective antigen(s) in a soluble rickettsial extract that we previously described.

Three other questions will continue to be addressed by this project: 1) What is the etiologic relationship to human illness of SFG agents other than R. rickettsii? 2) Are the other SFG serotypes unstable phenotypic variants of R. rickettsii? 3) If not, what is their ecologic role in determining the natural distribution of R. rickettsii and the focality of RMSF in the United States?

Publications:

Anacker, R. L. and Ormsbee, R. A.: Rickettsiae: general descriptions. In Seligson, D. (Ed.): Handbook Series in Clinical Laboratory Science. Section H. Virology and Rickettsiology Cleveland, CRC Press, Inc., 1978, vol. 1, part 2, pp. 329-360.

Philip, R. N., Casper, E. A., and Burgdorfer, W.: Current knowledge of the distribution of serotypes of spotted fever-group rickettsiae in the United States as determined by microimmunofluorescence. In: Proceedings VII International Congress of Infectious and Parasitic Diseases, Varna, Bulgaria, Oct. 2-6, 1978, 1978, pp. 500-509.

Philip, R. N., Casper, E. A., Burgdorfer, W., Gerloff, R. K., Hughes, L. E., and Bell, E. J.: Serologic typing of rickettsiae of the spotted fever group by microimmunofluorescence. J. Immunol. 121: 1961-1968, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00070-14 LMSF

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Biological Activities of Substances in Bordetella pertussis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. K. Bergman	Scientist Director	LMSF NIAID
OTHER:	J. J. Munoz	Res. Microbiologist	LMSF NIAID

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION
Pertussis Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	1.1	1.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to study the biological effects of substances from Bordetella pertussis with special emphasis on those substances which induce physiological and immunological responses in experimental animals. Areas of current investigation are: 1) ability of substances from B. pertussis to affect production of insulin by mouse pancreatic β-islet cells in primary tissue culture, 2) ability of pertussigen to induce production of unique peptides that may enhance circulatory shock, and 3) effect of substances from B. pertussis on insulin binding sites.

PHS-6040
(Rev. 10-76)

Project Description:

The purpose of this project is to investigate in experimental animals the biological effects of substances from Bordetella pertussis which produce physiological and immunological responses that are related to hypersensitivity and autoimmune phenomena.

Much of our previous work on the physiological and immunological effects of substances from B. pertussis has utilized in vivo techniques. The majority of the work has been done in mice and rats, and while the work has been informative, we have not had much insight into what was happening at the tissue or cellular level. During this past year, we have attempted to develop in vitro models that would allow us to investigate the effects of biologically active substances from B. pertussis on the pancreas and on insulin binding sites of plasma membranes.

Our attempts to cultivate mouse parotid salivary gland epithelial cells in tissue culture (as mentioned in last year's report) were unsuccessful. Since substances from B. pertussis have a profound effect on the pancreatic β-islets of rats (as reported by Ui et al. at the International Symposium on Pertussis, NIH, 1978), we decided to investigate the effects of our pertussigen preparations on mouse pancreatic β-islet cells in primary tissue culture. We now have a system which utilizes a trypsin-collagenase digest of neonate mouse pancreas and produces viable β-cells that grow in a medium containing TCM 199, NCTC 135 and fetal calf serum. The glucose concentration is adjusted to 16.5 mM. The β-cells seem to grow well in this system for 7 to 10 days and produce insulin as detected by a radioimmunoassay system. The α-cells from the islets do not appear to proliferate in the tissue culture since the production of α-amylase drops off rapidly. After 10 to 14 days, fibroblasts become predominant in the culture.

Since substances from B. pertussis are known to affect the pancreas, and pertussigen-treated mice and rats are much more susceptible to circulatory shock, we have made some preliminary tests for the production of a peptide in the ischemic pancreas of pertussigen-treated rats that might be analogous to the myocardial depressant factor (MDF) of Lefer (Fed. Proc. 37: 2734-2740, 1978). Results from thin layer chromatography (cellulose) and polyacrylamide gel electrophoresis have given some indication that there is a unique peptide in the plasma of pertussigen-treated rats following 1 hour of splanchnic ischemia that may be similar to the myocardial depressant factor, however we do not feel that these results have been definitive. We plan on investigating this question further by utilizing high performance liquid chromatography to purify and quantitate the plasma peptides.

We have also initiated some work to investigate whether or not pertussigen affects insulin binding sites on plasma membranes of mouse liver cells. We utilized the method of Ray (Biochim. Biophys. Acta 196: 1-9, 1970) to prepare plasma membranes and the methods of Caron et al. (Biochim. Biophys. Acta 512: 29-40, 1978) to measure insulin binding sites.

Preliminary results have not shown any difference in insulin binding between plasma membranes from pertussigen-treated mice and those from saline-treated mice.

Future work will investigate via in vitro methods the insulin production of mouse pancreatic β-islet cells following treatment with different substances from B. pertussis and also interactions with adrenergic agonists and antagonists. We will investigate whether or not pertussigen-treated rats release a unique peptide into the circulation during circulatory shock which may further complicate the shock syndrome. This will be investigated by the use of high performance liquid chromatography. We will also continue our efforts to measure the possible effects of pertussigen on insulin binding sites in both mice and rats.

Publications:

Bergman, R. K., Munoz, J. J., and Portis, J. L.: Vascular permeability changes in the central nervous system of rats with hyperacute experimental allergic encephalomyelitis induced with the aid of a substance from Bordetella pertussis. Infect. Immun. 21: 627-637, 1978.

Munoz, J. J., Bergman, R. K., and Robbins, K. E.: Comparison of the histamine hypersensitivity and the Limulus amoebocyte lysate tests for endotoxin activity. Infect. Immun. 22: 292-294, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00071-09 LMSF

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Purification and Activities of Pertussigen - a Substance from Bordetella pertussis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	H. Arai	NIH Visiting Fellow	LMSF NIAID
	R. K. Bergman	Scientist Director	LMSF NIAID
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	E. E. Ribi	Res. Chemist	LMSF NIAID

COOPERATING UNITS (if any)

S. M. Strain, Hamilton Biochemical Research Lab, Hamilton, MT

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Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION

Pertussis Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.9	4.0	1.9

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this project are to fully characterize and to study the activities of pertussigen, a substance from Bordetella pertussis, and to find the role that this and other substances play in the actions of B. pertussis. Methods to obtain pertussigen in highly purified form have been developed, and with these preparations we are studying their ability to protect mice from experimental pertussis. The effect that other substances from B. pertussis have on the protective effect of pertussigen is presently under investigation. Particular attention has been given to the mechanisms by which pertussigen enhances the production of immunoglobulin E (IgE) in mice. These studies indicate that pertussigen may act directly on the lymphoid cells responsible for antibody production.

PHS-6040
(Rev. 10-76)

Project Description:

The primary objectives of this project are to characterize the chemical nature of pertussigen and other biologically active substances of the Bordetella pertussis cell and to uncover the mechanisms of their biological activities. The ultimate practical aim is to develop a pertussis vaccine free of toxicity.

1. Purification of pertussigen (Munoz, Arai and Sone). In the 1978 report we described a method of obtaining highly purified preparations of pertussigen. This method employed Triton X-100 which we found difficult to remove from the purified material. Furthermore, the purified material was not stable when frozen and thawed. For these reasons we continued our efforts to find better methods of purification. Two methods have been tried with promising results. Phenyl sepharose absorbs pertussigen and it can then be eluted with a high pH buffer. This simple step can then be followed by filtration in a BioGel A.5 column and by ultracentrifugation in a glycerol gradient to produce a highly active purified pertussigen. The other method involves the use of affinity chromatography columns made by conjugating either haptoglobulin or specific anti-pertussigen serum to Sepharose beads and then eluting pertussigen by an appropriate buffer. This latter method is promising and is presently under extensive experimentation.

2. Studies on fimbrial hemagglutinins (FHA) (Arai and Munoz). B. pertussis produces two distinct hemagglutinins, one which is identical to pertussigen and the other called fimbrial hemagglutinin. According to Sato and Arai, FHA protects mice from experimental pertussis. We have found that under the conditions normally used at RML to grow B. pertussis (shaking cultures), FHA is not found in 3- to 5-day-old cultures. However, under the conditions used by the Japanese workers (stationary incubation in shallow layers of media), FHA is produced. Employing stationary cultures of B. pertussis, FHA was purified and crystallized by a simple procedure which involved passing 5-day-old culture supernatant fluids through a CM-Sepharose CL-6B column equilibrated in 20 mM phosphate buffer pH 6.5. After washing the column with 20 mM phosphate buffer, FHA was eluted with 0.5 M NaCl in 20 mM phosphate buffer containing 5% ethanol. The active fractions were pooled, concentrated by vacuum dialysis and passed through Sepharose 6B columns equilibrated in 50 mM Tris-HCl buffer containing 1 M NaCl and 5% ethanol. The active fractions were pooled and concentrated by vacuum dialysis. During this concentration, crystallization of FHA occurred. These crystals were made up entirely of amino acids and had, when dissolved, a specific activity as high as that of uncrystallized purified FHA.

3. Activities of purified FHA and pertussigen (Munoz and Arai). Although FHA was highly purified, it still was able to sensitize CFW mice to histamine at doses of 2 to 5 µg per mouse. This crystalline preparation also protected mice from experimental infection with B. pertussis at similar doses. Since the ability to sensitize mice to histamine is a property of pertussigen, it was thought that crystalline FHA was still

contaminated with pertussigen. So we proceeded to remove pertussigen from these preparations. This was accomplished by column chromatography in BioGel in the presence of Triton X-100. Preparations of FHA free of pertussigen did not protect mice from experimental infection or sensitize them to histamine at a dose of 25 μg.

Pertussigen purified by the method described in last year's report or by methods described here do not contain FHA, but are highly active in sensitizing mice to histamine (SD_{50} about .01 μg of protein) and to protect them from infection (PD_{50} 1 to 2 μg).

4. Preparation of specific antisera to FHA and pertussigen and passive protection test with these sera (Munoz and Arai). Antisera to crystalline FHA and purified pertussigen were made by repeatedly immunizing rabbits with these substances either mixed with complete Freund's adjuvant (for FHA) or mixed with erythrocyte stromata. The antisera obtained were not specific to FHA, but were made specific by passing them through an affinity column containing the appropriate antigen, and then eluting the adsorbed antibody by appropriate buffer. The specificity of the eluted antibodies was determined by gel diffusion tests against the purified FHA and pertussigen. Passive protection tests were performed with these antisera. Anti-pertussigen serum protected mice from intracerebral infection while the anti-FHA did not.

5. Relationship among pertussigen, FHA and agglutinogens (Munoz and Arai). The relationship of the various agglutinogen factors to pertussigen and FHA has not been defined. We have studied these relationships and have arrived at the following conclusions:

a) Pertussigen and FHA are not associated with agglutinogens 2, 3, 4, 5, 6, because we can obtain these substances from B. pertussis agglutinogen types 1, 3, 6 or 1, 2, 4.

b) Pertussigen and FHA are two distinct substances which are produced by all smooth cultures of B. pertussis thus far tested. B. bronchiseptica and B. parapertussis produce smaller amounts of FHA but not pertussigen.

c) Agglutinogen 1 is not related to FHA because B. parapertussis and B. bronchiseptica produce FHA but not agglutinogen 1.

d) Pertussigen is different from agglutinogen 1 because anti-pertussigen serum fails to agglutinate cells that are strongly agglutinated by factor 1 antiserum.

6. Adjuvant action of pertussigen (Sadowski and Munoz). One of the many biological properties of pertussigen is its ability to increase antibody production to antigens given with it. An unusual characteristic of this

adjuvant action is that the IgE response is almost selectively increased. Understanding the mechanism(s) by which pertussigen exerts this effect may reveal how IgE production is regulated and thus may lead to a better understanding of the allergic response in man.

The following observations have been made:

a) Relationship of pertussigen to endotoxin. Since the preparations of pertussigen used were not free of endotoxin, it was necessary to rule out the participation of endotoxin in stimulation of IgE. Pertussigen acts as an adjuvant for stimulation of IgE in C57BL/10ScN mice which are unresponsive to endotoxin. Doses of Escherichia coli endotoxins up to 100 μg (which also contains endotoxin protein) do not stimulate an IgE response in these mice.

b) Time at which pertussigen acts. Pertussigen acts as well in stimulating an IgE response when administered 3 days prior to the antigen as when administered with the antigen.

c) Effect of Concanavalin A. In contrast to the last observation, Concanavalin A, which also induces IgE stimulation when given with the antigen, does not stimulate production of IgE when given 3 days before the antigen. Most adjuvants will not stimulate Ig response to an antigen when administered prior to the antigen, and often they will suppress antibody response. Preliminary work indicated that pertussigen given 3 days prior to antigen may not be as effective in stimulating IgM production as when it is given simultaneously with the antigen.

d) Role of suppressor cells. The ability of pertussigen to act as an adjuvant for IgE production when given 3 days prior to antigen is similar to the effect of cyclophosphamide. The action of cyclophosphamide is thought to be due to the elimination of suppressor T cells that are responsible for exerting a negative control over IgE synthesis.

e) Effect of anti-pertussigen sera. Antisera capable of inhibiting the histamine sensitizing action of pertussigen also inhibit its ability to act as an adjuvant. When pertussigen is administered 3 days prior to antigen and antiserum is given with the antigen, the adjuvant effect of pertussigen is still suppressed. However, if antiserum to pertussigen is administered 3 days after antigen and pertussigen, the IgE stimulation is not inhibited.

f) Irradiation studies. The ability of pertussigen to stimulate IgE response can be transferred into irradiated recipients by spleen cells from mice which had received pertussigen 3 days prior to the removal of the spleens. The irradiated recipient mice, when given antigen, produce IgE with specificity to the antigen. This may mean that pertussigen was transferred with the spleen cells although other explanations may be possible.

g) SDS acrylamide gel electrophoresis studies. In spleen cells of mice receiving radioiodinated pertussigen 3 days prior to removing the spleens, a component of the pertussigen preparation was demonstrated by extracting the cells with detergent and then treating the extract with anti-pertussigen serum. The material precipitated by the antiserum showed, by analysis on SDS-PAGE gels, one radioactive polypeptide of about 18,500-19,000 molecular weight.

7. Miscellaneous experiments (Arai and Munoz). Pertussigen increases the leukocyte count in mice, but in our hands the increase in the number of leukocytes has been generally of a lower order of magnitude than that reported by other workers. It was possible that these differences were due to the source of blood to perform the leukocyte counts. Indeed, we found that counts performed with blood obtained by snipping the end of the tail were significantly higher than those counts performed with blood obtained from the infraorbital sinus. This difference was probably due to hemoconcentration at the tip of the tail. When blood was obtained from the tail, after allowing blood to flow freely, the leukocyte counts were similar to those obtained from the infraorbital sinus.

During the following fiscal year we will continue our efforts to fully characterize pertussigen and to find its mechanisms of action, especially with respect to its ability to stimulate IgE and to protect mice from experimental pertussis. We will study the role that various purified B. pertussis antigens play in protection, and will study ways of detoxifying the protective antigen to see if we can develop a vaccine with fewer side effects than the presently available pertussis vaccines. Much time will be devoted in obtaining sufficient quantities of pertussigen for chemical identification purposes.

Publications:

Munoz, J. J. and Bergman, R. K.: Mechanism of action of pertussigen, a substance from Bordetella pertussis. Microbiology 1979 193-197, 1979.

Munoz, J. J. and Cole, R. L.: Extraction of pertussigen from Bordetella pertussis with aid of Triton X-100. IRCS Med. Sci. 7: 218, 1979.

Arai, H. and Munoz, J. J.: Leukocyte counts in blood obtained from tail and infraorbital sinus bleedings in normal and pertussigen-treated mice. IRCS Med. Sci. 7: 306, 1979.

Wardlaw, A. C., Parton, R., Bergman, R. K., and Munoz, J. J.: Loss of adjuvanticity for hyperacute EAE and for reaginic antibody production in a phenotypic variant of Bordetella pertussis. Immunology 37: 539-545, 1979.

Publications: (cont'd)

In press:

Munoz, J. J. and Bergman, R. K.: Biological activities of Bordetella pertussis. Proceedings of International Pertussis Conference, Bethesda, MD, Nov. 1978.

Arai, H. and Munoz, J. J.: Purification and crystallization of fimbrial hemagglutinin from Bordetella pertussis. Infect. Immun.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00076-21 LMSF

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Mechanisms of Immunopotentiation by Components of Microbes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. A. McLaughlin	Senior Staff Fellow	LMSF NIAID
OTHER:	E. E. Ribi	Res. Chemist (Phys. Chem.)	LMSF NIAID
	S. M. Schwartzman	Senior Staff Fellow	LMSF NIAID
	J. L. Cantrell	Senior Staff Fellow	LMSF NIAID
	K. B. Von Eschen	Staff Fellow	LMSF NIAID

COOPERATING UNITS (if any) Dr. E. P. Goldberg, Dept. Engineering, Univ. of Florida, Gainesville; Dr. C. H. Granatek, M.D. Anderson Tumor Institute, Houston, TX; Dr. R. Toubiana, Gif-sur-Yvette, France; Dr. G. Jones, Syntex Corp., Palo Alto, CA.

LAB/BRANCH
Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION
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INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.85	1.25	1.6

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to delineate, at the cellular and molecular levels, the mechanism of action of microbial components in immunopotentiation. In the past year, two major efforts have been made: 1) elucidating the nature of biological responses to treatment of tumors with synthetic analogs of mycobacterial cell wall components combined with endotoxic extracts obtained from Salmonella typhimurium Re mutants, and 2) establishing the structural features of analogs of adjuvant dipeptides required for antitumor activity when combined with trehalose dimycolate.

PHS-6040
(Rev. 10-76)

Project Description:

In general, our major objective is elucidating the structural requirements and mechanism of action of immunopotentiating components isolated from microbes.

Previously we had shown that mycobacterial cell wall skeleton, when combined with endotoxic extracts from Salmonella typhimurium rough mutants (Re glycolipid), produced synergistic, not additive, tumor-regressive responses. The mechanism of this synergy was found not to be a consequence of interaction of the cell wall skeleton with endotoxin molecules. This was unlike the synergistic response seen when trehalose dimycolate was combined with Re glycolipids wherein we showed that the trehalose dimycolate enhanced the interaction of endotoxin molecules with oil droplets of the oil-in-water emulsions used as a therapeutic vehicle. We had not established the minimal structural requirement of cell wall skeleton to produce the synergistic antitumor activity observed upon combination with Re glycolipid. Last year we proposed that the adjuvant dipeptide, N-acetyl-muramyl-L-alanyl-D-isoglutamine might represent at least one, if not the only, minimal structural unit in cell wall skeleton for the synergy observed with endotoxin. Indeed the adjuvant dipeptide did replace the cell wall skeleton in producing synergistic antitumor activity in combination with endotoxic extracts. The synthetic molecule and Re glycolipid produced high incidences of tumor regression of transplanted dermal tumors in guinea pigs. However, only upon addition of trehalose dimycolate (provided by Dr. R. Parker, Hamilton, MT, under contract with NIAID) were the treated animals cured of both the dermal and metastatic tumors. Another synthetic preparation (provided by Drs. I. Azuma and Y. Yamamura), 6-O-mycoloyl-N-acetyl-muramyl-L-alanyl-D-isoglutamine in combination with Re glycolipid, produced synergistic tumor-regressive activity against both the dermal and metastatic tumors. All of these preparations were active only when presented to the tumor-bearing animal in an oil-in-water emulsion constructed to maximize the interaction of the synthetic preparations with the oil phase of the emulsions. The Re glycolipid was effective in the aqueous phase of the emulsion. Thus, we have now defined the structural features of the BCG cell wall or cell wall skeleton responsible for the potent antitumor activity of these preparations when combined with endotoxic extracts. The requirement for a covalent or noncovalent lipidation of the adjuvant dipeptide for eliciting antitumor activity against metastatic tumors prompts one to speculate about the mechanism of this phenomenon. Conceivably the complexing of biologically active molecules (adjuvant dipeptide) with oil droplets via a lipid rich molecule such as mycolic acid or mycolic acid esters would produce a kind of depot effect in tissues into which the oil-in-water emulsion was injected. Another reasonable hypothesis is that the muramyl dipeptide-oil-droplet complex was preferentially processed by a specific population of cells in the afferent or efferent arms of the immune system. Experiments conducted with radiolabeled cell wall skeleton suggest that the later mechanism may be more important than a slow-release (depot) effect at the site of injection. We found that radiolabeled cell wall skeleton suspended in saline and injected into dermal tumors remained in the tumors and in other tissues for a period

of time equal to cell wall skeleton combined with trehalose dimycolate and suspended in an oil-in-water emulsion. Cell wall skeleton in saline has no tumor regressive activity whereas cell wall skeleton combined with trehalose dimycolate in an oil-in-water emulsion is a very effective tumor-regressive preparation. Additional experiments are underway whereby we will utilize radiolabeled muramyl dipeptide in similar studies to determine the distribution of muramyl dipeptide in vivo following intratumor injections. It may be that a depot effect is not critical to expression of the biological activity of these naturally occurring and synthetic molecules, but what is important is the nature of their presentation to the cells involved in that expression.

As an extension of the finding that N-acetyl-muramyl-L-alanyl-D-isoglutamine possessed some of the biological activity of cell wall skeleton, we initiated a collaborative study with Dr. G. Jones, Syntex Corporation, Palo Alto, CA. We have found analogs of the original adjuvant dipeptide which are approximately 100 times as effective in producing tumor regression as the parent compound. All of the newly described analogs are active only when combined with trehalose dimycolate in the construction of oil-in-water emulsions utilized as therapeutic vehicles for intratumor injections. In collaboration with Dr. Raoul Toubiana, we have found that synthetic analogs of trehalose dimycolate, such as trehalose desoxypalmitopalmitate and glycerol monomycolate, act in concert either with endotoxic extracts or the synthetic muramyl dipeptides to produce significant incidences of tumor regression. Thus, we have successfully simulated some of the biological activities of mycobacterial components through the use of synthetic substitutes. These synthetic preparations will hopefully facilitate the elucidation of the mechanism of action of microbes in modulating immune responses and in pathogenesis of disease processes.

An important step in studying the mechanism of action of muramyl dipeptides at the molecular level is the synthesis of radiolabeled muramyl dipeptide. Dr. Schwartzman successfully synthesized ^{3}H-labeled adjuvant dipeptide, but the procedure utilized resulted in relatively low yields of material. Another labeling procedure is in progress which will use ^{3}H-acetic anhydride and require fewer steps. ^{13}C-labeled muramyl dipeptide to be synthesized by Dr. Schwartzman will be another useful tool in studying the mechanism of action of this important compound.

The future course of this project will be concentrated in two major directions: 1) the elucidation of the biochemical mechanism of action of muramyl dipeptides, and 2) construction of the most active adjuvant-antigen-carrier complexes for treatment and prevention of infectious and neoplastic diseases. Dr. Schwartzman has proposed that muramyl dipeptide be conjugated to suitable carriers, such as Ficoll or polyglutaraldehyde, as promising candidates in construction of active adjuvant-carrier complexes. Covalent coupling of trehalose monoesters to antigens of interest will be a project

undertaken by Dr. R. Toubiana. Covalent linkage of muramyl dipeptides to trehalose dimycolate or to antibodies directed against tumor cells are other avenues to be pursued by Dr. Schwartzman.

We now have synthesized minimal structural components of mycobacteria required for elicitation of certain biological responses conceivably important in disease processes and potentially useful in disease control. These synthetic substances will greatly simplify the task of visualizing the biochemical steps leading to the phenomenon now called adjuvant activity or tumor-regressive activity.

Publications:

McLaughlin, C. A., Ribi, E. E., Goren, M. B., and Toubiana, R.: Tumor regression induced by defined microbial components in an oil-in-water emulsion is mediated through their binding to oil droplets. Cancer Immunol. Immunother. 4: 109-113, 1978.

McLaughlin, C. A., Bickel, W. D., Kyle, J. S., and Ribi, E.: Synergistic tumor regressive activity observed following treatment of line-10 hepatocellular carcinomas with deproteinized BCG cell walls and mutant Salmonella typhimurium glycolipid. Cancer Immunol. Immunother. 5: 45-52, 1978.

McLaughlin, C. A., Hargrave, S. L., Bickel, W. D., and Ribi, E.: Synergistic activity of components of mycobacteria and mutant Salmonella in causing regression of line-10 tumors in guinea pigs. Cancer Res. 39: 1766-1771, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00077-23 LMSF

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Structure and Biological Activity of Endotoxins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. B. Von Eschen	Staff Fellow	LMSF NIAID
	E. E. Ribi	Res. Chemist (Phys. Chem.)	LMSF NIAID
OTHER:	E. C. B. Milner	Student Scientist	LMSF NIAID
	C. A. McLaughlin	Senior Staff Fellow	LMSF NIAID
	J. L. Cantrell	Senior Staff Fellow	LMSF NIAID
	S. M. Schwartzman	Senior Staff Fellow	LMSF NIAID
	J. C. Williams	Senior Asst. Scientist	LMSF NIAID
	J. Sasaki	Visiting Associate	LMSF NIAID

COOPERATING UNITS (if any) Dr. R. A. Parker and S. M. Strain, Hamilton Biochemical Res. Lab., Hamilton, MT; Dr. J. A. Rudbach, Dr. M. J. Nakamura, and S. Wells, University of Montana, Missoula; and Dr. R. Wheat, Duke University, Durham, NC.

LAB/BRANCH

Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.7	1.1	2.6

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The main objective of this project is to determine the minimal chemical and structural properties of bacterial endotoxins necessary to elicit several biologic and immunologic responses. The purpose is to identify, by fractionation of different endotoxins and chemical, physical, and biological analyses of the individual fractions, the component(s) of the macromolecular complex responsible for the antitumor effects of some endotoxins. Techniques are being developed to assess the cellular nature of the immunomodulatory activities of different endotoxic materials. Also, this project continues to perform standard bioassays of endotoxic materials contributed by several members of the LMSF.

PHS-6040
(Rev. 10-76)

Project Description:

Bacterial endotoxins, complex macromolecules extracted from gram-negative bacteria, possess a great variety of biological and immunological activities. Studies conducted in the Laboratory of Microbial Structure and Function continue to stress the potential use of endotoxins as potent immunotherapeutic reagents for the treatment of certain neoplastic diseases. To be effective in the syngeneic guinea pig line-10 tumor model, endotoxins have to be combined with certain components of mycobacteria, such as trehalose dimycolate, cell wall skeleton or adjuvant dipeptide (see annual reports ZO1 AI 00078-07 LMSF, Ribi; ZO1 AI 00076-21 LMSF, McLaughlin; and ZO1 AI 00087-02 LMSF, Cantrell). Whereas the mycobacterial components responsible for tumor immune responses are now defined and have been chemically synthesized, the endotoxic extracts we are utilizing remain complex, poorly defined enigmas. Some progress in the fractionation of these extracts was made in the last year. Dr. McLaughlin successfully used equilibrium cesium chloride density gradient centrifugation to separate contaminating substances, such as nucleic acids, from endotoxin-rich fractions. We found that this technique demonstrated the presence of phospholipid-endotoxin complexes in a variety of endotoxic extracts. Interaction of endotoxin with phospholipids obtained from a variety of bacteria caused a marked alteration in the density of the endotoxin. The means of extraction produced varying degrees of complexation as a function of the amount of phospholipid contaminating the endotoxin-rich extracts. These findings suggest that a re-evaluation of the proposed chemical structure of endotoxin is required. Considerable effort will be needed to establish the purity of endotoxic extracts before assigning structural details to a general formula for endotoxin (McLaughlin).

The use of purified cell walls from Re mutant strains of gram-negative bacteria rather than whole cells for the extraction of endotoxic material appeared to be a step in the right direction. Endotoxic extracts prepared from cell walls, particularly EDTA-treated cell walls, in contrast to those obtained from whole cells, were soluble in both water and organic solvents, apparently as a result of dissociation of glycolipid-phospholipid complexes which had been held together by polyvalent cations. A long standing problem of endotoxin solubility has, therefore, been solved that will facilitate in vitro and in vivo experimentation as well as fractionation of endotoxic material.

When endotoxic chloroform-methanol extracts of cell walls were chromatographed through columns of microparticulate silica gel, the procedure used to prepare B4 (see annual report ZO1 AI 00078-07 LMSF, Ribi), the major component, after being rechromatographed, was rendered essentially nontoxic while pyrogenicity was retained. Studies on the immunostimulating activities of the extract before and after detoxification also showed discrete differences. Lymphocytes from genetic endotoxin responder (C3HeB/FeJ) and nonresponder (C3H/HeJ) strains of mice were used. In general, the starting material and nontoxic fractions were potent mitogens

and polyclonal B cell activators for C3HeB/FeJ mice. For C3H/HeJ mice, the starting extract was again mitogenic and a polyclonal B cell activator, but the nontoxic fraction was essentially inactive in these respects. Finally, gel diffusion analysis revealed a line of complete identity between these samples. The results showed that certain typical endotoxic properties can be selectively altered by purification of endotoxic glycolipid on silica gel columns.

Other studies, done in collaboration with Mr. Steven Wells and Dr. M. J. Nakamura, examined the chemical and biological characteristics of endotoxins extracted from Yersinia enterocolitica. Although this gram-negative organism has been gaining importance as a causative agent in certain enteric and systemic infections, little is known about the possible role that endotoxin plays in its pathogenicity. Biologically active endotoxins were extracted from both a pathogenic strain (serotype O:8, isolated at necropsy from a human spleen) and a nonpathogenic strain (serotype O:17, isolated from water) of Y. enterocolitica. The chemistry and biological potency of these endotoxins were similar to those of a standard endotoxin extracted from Escherichia coli. In addition, the relative yield of endotoxin was considerably higher from Yersinia organisms cultivated at 25°C than at 37°C. This latter finding may be important in that organisms grown at 25°C were reported to be more pathogenic than cells grown at 37°C. Also, based on similarities in endotoxins, a taxonomical relationship between Yersinia and at least one other Enterobacteriaceae was established. Mr. Wells wrote his M.S. thesis in microbiology from this work and is currently writing a manuscript for publication.

Another project of primary interest has been the interaction and functional stimulation of lymphocytes with bacterial endotoxin. Historically, endotoxins were reported to be potent stimulators of primarily bursa equivalent lymphocytes (B cells). Experiments performed by Mr. Eric Milner, a Ph.D. student working in my laboratory, provided new and exciting information concerning the interaction of endotoxins with thymus-derived lymphocytes (T cells). A system of in vitro proliferation was used to assess the effects of endotoxin on the in vivo induction and modulation of antigen-specific T cells. It was found that nylon wool nonadherent lymph node cells from C57BL/6J mice immunized with endotoxin gave strong proliferative responses upon in vitro stimulation with endotoxin whereas the responses of T cells from unimmunized mice were weak or absent. Since endotoxin is known to be a nonspecific mitogen for B cells, it was of prime interest to characterize the cellular nature of the endotoxin-specific response. Results from several experiments suggested that the cells responding to endotoxin were T cells, including: 1) endotoxin-specific responses were generated in nylon wool nonadherent cells which were 95% positive for theta antigen, 2) the responses were abolished by treatment of the cells with anti-Thy 1.2 serum plus complement, 3) the responses were not diminished by treatment of the cells with anti-Ig serum plus complement, 4) virtually all responding cells stained

positively with fluorescent anti-Thy 1.2 serum, and 5) endotoxin-specific responses were not obtained in nylon wool-purified lymph node cells from athymic nude mice.

Using this system, reciprocal cross-reactivity was observed between proliferative responses of T cells primed to endotoxin from E. coli or Salmonella minnesota. These data indicated that the T cell response was directed to antigens shared by endotoxins from different species of gram-negative bacteria.

Antigen-specific T cell responses to endotoxin were not detected in C57BL/10ScN mice which are genetic nonresponders to endotoxin. This discovery suggested that the unresponsiveness of cells from endotoxin nonresponder strains of mice, reported to be restricted to B cells and macrophages, was also extended to T cells.

The antigen specific proliferative system was also used to study the adjuvant activity of bacterial endotoxins. Lymph node T cells from mice immunized with a mixture of ovalbumin plus endotoxin gave greatly enhanced responses upon in vitro stimulation with ovalbumin, as compared to T cells from mice immunized only with ovalbumin. Endotoxin did not act as an adjuvant in genetic nonresponder strains of mice. These are the first observations on the capacity of endotoxin to potentiate the generation of antigen-reactive T cells in vivo.

We would like to re-emphasize the importance of the studies on the specific and nonspecific interaction of endotoxin with T cells with respect to the following points. 1) The discovery of endotoxin specific T cells provides the potential that such T cells participate in immune responses to endotoxins. Based on this finding, we would like to recommend a re-evaluation of the paradigmatic idea that endotoxin is purely a "T cell independent antigen." 2) The capacity of endotoxin to potentiate T cell responses to other antigens may provide, in part, an explanation for the immunotherapeutic activities of endotoxin described in this and accompanying progress reports.

This work, which constitutes a major portion of Mr. Milner's Ph.D. thesis, was presented at the 1979 Montana Academy of Sciences Meeting ("best paper by a graduate student award") and also at the annual meeting of the American Society for Microbiology. Mr. Milner is currently writing 2 manuscripts on these findings.

The capacity of bacterial endotoxin (ET) to inhibit the growth of tumors in mice genetically responsive or unresponsive to the diverse biological effects of ET was also studied. Admixtures of 10 to 250 μg of ET extracted from E. coli 0113 and viable MC-93 tumor cells resulted in a dose dependent reduction in the incidence of tumors when the mixtures were injected into C3HeB/FeJ mice (ET responders). This suppression of tumor growth was not the result of a direct cytotoxic effect of the ET for the MC-93 tumor cells and was reduced markedly by base hydrolysis of the ET. Similar doses of ET

failed to reduce significantly the incidence of tumors in C3H/HeJ mice (ET nonresponders). In addition, ET suppressed the growth of EL-4 tumors in C57BL/10Sn mice (ET responders) but had no effect on the incidence of tumors in the ET nonresponder C57BL/10ScN mice. Results of experiments with 3 relatively nonendotoxic extraction products from gram-negative bacteria, acetone-chloroform precipitate (ACP), free lipoprotein (BLP), and endotoxic protein (EP) showed that antitumor activity did not correlate exclusively with mitogenicity or toxicity. ACP and BLP were mitogenic for both C3HeB/FeJ and C3H/HeJ mice but did not suppress significantly the growth of MC-93 tumors in these animals. Also, EP was 175 times less toxic than the standard ET used for tumor suppression and still suppressed 75 to 90% of the tumors in the C3H mice. Subsequent experiments showed that 1) 64% of the mice which suppressed primary tumors rejected a second injection of homologous tumor cells, 2) tumor immune lymphocytes were detected by a modified Winn assay in the spleens of mice which rejected primary tumors, and 3) endotoxin did not suppress the growth of tumors in congenitally athymic nude mice. In general, these data indicated that, in genetic responder mice, the antitumor effectiveness of endotoxin depended upon elicitation of a host-mediated response, possibly involving T cells.

Future plans for this project include several experiments which will hopefully describe further the relationship between the structure and function of bacterial endotoxins. Toxic and nontoxic purification products from endotoxic glycolipid will be analyzed for additional biological activities including the capacity of these materials to suppress murine tumors, act as adjuvants in conventional antibody systems and also in the generation of antigen specific T cells. Additionally, attempts will be made to restore the activity of B4-rechrom by combining this material with other bacterial and synthetic products.

The antigen specific T cell proliferative assay, in conjunction with the judicious selection of endotoxins extracted from several species of gram-negative bacteria (including those from defined mutants of S. minnesota), will be used to study the fine specificity of T cell responses to endotoxin antigens. More extensive experiments on the adjuvant effect of endotoxin on T cell responses to other antigens will be done including the effect of stimulating T cells with endotoxin before or after exposure to antigen. Also, this assay system will be used to evaluate the level of antitumor immune T cells in mice which have undergone endotoxin-induced suppression of primary tumors.

With respect to the suppression of tumors, we hope to elucidate more clearly the cellular requirements for the antitumor effects of endotoxins. For these studies, we plan to adoptively transfer select subpopulations of lymphoid cells from endotoxin responder mice to X-irradiated endotoxin nonresponder recipient mice. These reconstituted mice will then be injected with tumor cells and endotoxin. Also, we plan to characterize the cellular nature of the antitumor lymphocytes found in tumor immune mice.

Finally, experiments have been started on the capacity of endotoxin to facilitate, in vitro, the generation of tumor immune lymphocytes. If successful, these experiments may provide us with a means of capitalizing on the immunopotentiating activity of endotoxin without exposing the host animal to the deleterious side effects endotoxin has in vivo.

Publications:

In press:

Winters, W. D., David, E., and Ribi, E.: Dichotomy of endotoxin action during adenovirus replication in human cells. J. Gen. Virol.

Winters, W. D., David, E., and Ribi, E.: Effects of bacterial endotoxins on human adenovirus. J. Gen. Virol.

Appendix 1. Contract NO1 AI 72525. Production of P3 and Isolation, Fractionation, and Purification of Lipopolysaccharides (Endotoxins), Hamilton Biochemical Research Laboratory, Hamilton, Montana.

Total Man Years: 3 Professional: 0.7 Other: 2.3

Annual Funding: $55,995.93

This project was designed, in part, to study and develop methods for the isolation, fractionation, and purification of bacterial endotoxins. During the reporting year, the contractor has supplied the Laboratory of Microbial Structure and Function with about 500 mg of P3 and about 300 mg of endotoxin which was freed of peptide contaminants (B4) by pressure elution chromatography through columns of microparticulate gel. These refined compounds have served as invaluable baseline materials for numerous tumor immunotherapeutic experiments referred to in four individual annual reports of Laboratory of Microbial Structure and Function.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00078-07 LMSF

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Microbial Components in Cancer Immunotherapy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. E. Ribi	Acting Chief	LMSF NIAID
OTHER:	C. A. McLaughlin	Senior Staff Fellow	LMSF NIAID
	J. L. Cantrell	Senior Staff Fellow	LMSF NIAID
	K. B. Von Eschen	Staff Fellow	LMSF NIAID
	S. M. Schwartzman	Senior Staff Fellow	LMSF NIAID

COOPERATING UNITS (if any) Dr. B. Zbar, NCI; Dr. W. Brehmer, Robert Koch Inst., Berlin; Dr. H. Huckauf, Med. Clinic, Berlin; Drs. S. Richman and C. R. Granatek, M.D. Anderson Hosp., Houston, Dr. G. Vosika, U. of Minn., Minneapolis; Dr. P. Minden, Natl. Jewish Hosp., Denver; Dr. R. Parker and S. M. Strain, Hamilton Biochem.Lab.

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Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION
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NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.15	1.05	3.1

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☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major objectives of this project continue to be the identification of nonviable, chemically defined microbial components that are efficacious antitumor agents and to develop and supply such preparations in stable, standardized form for both experimental and clinically applied immunotherapy. The intent of the research is to circumvent the use of infectious agents and, by preparing synthetic analogues, provide alternative substances that are not antagonistic or that do not produce undesirable biological responses.

PHS-6040
(Rev. 10-76)

Project Description:

The major objective of this project is to develop clinically useful immunostimulants for tumor regression. Delineation of the mechanisms of immunomodulation by microbial components has been the goal of members of the Molecular Biology Section (see annual reports submitted individually by Drs. McLaughlin, Cantrell and Von Eschen).

The transplantable line-10 hepatocellular carcinoma of guinea pigs developed at NCI has been used as a model for the study of weakly antigenic malignant tumors. When 6-day-old (10 mm in diameter) tumors were inoculated intralesionally with viable BCG, a significant proportion of such tumors regressed permanently and, as subsequently reported, these results could be equaled by treatment with nonviable mycobacterial cell wall components associated with oil droplets in saline emulsion. They included purified cell walls and deproteinized, delipidated cell walls, designated cell wall skeleton (CWS). Either of these products, when combined with trehalose dimycolate (P3), a lipoid component removed from the cell walls which is also known as purified cord factor, was highly effective. (P3 was supplied by Dr. R. Parker, Hamilton Biochemical Research Laboratory under contract N01 AI 72525). We also reported that a high cure rate was obtained with materials unrelated to tubercle bacilli, provided they were combined with P3. Particularly impressive in this respect were chloroform-methanol extracted endotoxic glycolipids (ReGl) from O antigen-deficient (Re) mutant strains of Enterobacteriaceae which provided up to 100% cures.

Whereas highly endotoxic lipopolysaccharide (LPS) extracts from all wild type strains so far tested failed to cause tumor regression, acid hydrolysis of Serratia marcescens led to residual fractions (RESI) which serologically cross-reacted with ReGl samples and provided a cure rate of 90%. This RESI was essentially nonpyrogenic and 100 times less lethal for laboratory animals than potent endotoxins, indicating that there was no correlation between the antitumor property and endotoxicity. Endotoxic glycolipids extracted from Re mutant bacteria with a monophasic mixture of phenol, chloroform and petroleum ether (Galanos et al.) were highly endotoxic but relatively ineffective in regression line-10 tumors. This also indicated that the most important ingredient was not endotoxin itself but rather some associated component(s) that was extracted with the glycolipid. In addition, we noted that the lower efficacy of such extracts was paralleled by a reduction of the content of muramic acid, alanine, and glutamic acid, which are characteristic components of bacterial cell wall peptidoglycan. Reduction of amino acid content of ReGl by microparticulate gel chromatography or by treatment with Triton X-100 significantly lowered its ability to bring about tumor regression without affecting endotoxicity. The chromatographically refined ReGl was designated B4. We considered that precursor or autolysis products of the peptidoglycan moiety of CWS may have been co-extracted with the endotoxic glycolipids. Indeed, antitumor activity to B4 could be restored by the addition of synthetic N-acetyl-muramyl-L-alanyl-(L-seryl)-D-isoglutamine (MDP), which is considered the minimal structural unit responsible for the adjuvant

action of microbial cell walls and CWS. The tumor-regressive activity of B4 could be also restored by the addition of a nontoxic lipoid side fraction (ACP) recovered during the isolation of ReGl which contained a small amount of peptidic substances or, as described in Dr. Cantrell's progress report (Z01 AI 00087-02), by the addition of Braun's lipoprotein known to contain covalently bound MDP moieties.

We found that lowering the dose of MDP from 150 µg to 1.5 µg still provided a cure rate of at least 90% when combined with 15 µg of B4. This small amount of peptidic material is of the order of magnitude (about 0.1%) of the amount of amino acids removed from ReGl during the preparation of B4. Skin testing data had indicated that delayed type hypersensitivity developed against the endotoxin-associated antigens as well as specific tumor antigens. MDP may act as an adjuvant to an antigen(s) which may have cross-reactive determinants associated with the endotoxin itself. Such antigens appear to be cryptic or sterically hindered from being effective in polysaccharide-rich LPS from wild type bacteria but are exposed in polysaccharide-deficient ReGl from Re mutant bacteria. It remains to be determined whether this cross-reactive antigen is identical with that shared by CWS, ReGl, the line-10 tumor cell and certain human tumors, such as malignant melanoma, as observed by Dr. Christine Granatek and Dr. Percy Minden. If so, cross-reactive antigens shared by microorganisms and tumor cells, and which are absent or cryptic in normal cells, may represent the immune determinants against which MDP, in concert with P3, augments antitumor immunity. Although there is no direct correlation between endotoxic potency and tumor regressive activity, our findings indicate that a low level of toxicity may be required to obtain optimal levels of tumoricidal action.

From a practical point of view, the findings reported here lend merit to the continued search for products that synergistically enhance the action of cell walls, CWS, or MDP but have low endotoxicity. It was logical, therefore, to examine the efficacy of combinations of clinically tested agents, such as Pseudomonas vaccine (Pseudogen), which is a registered product prepared by Warner-Lambert/Parke-Davis, and is an LPS-containing extract whose relatively low endotoxicity was, in fact, comparable to that of RESI from S. marcescens. Unexpectedly, we found that when a combination of Pseudogen, ReGl or B4 (150 µg), and MDP (150 µg), even in the absence of P3, was inoculated into established dermal line-10 tumors, a significant number of the animals died, probably of endotoxic shock. All surviving animals suffered severe but temporary lethargy. For yet unexplained reasons, Pseudogen was even more active in this respect than ReGl. These effects did not depend upon the presence of malignant tissue. When administered alone intradermally at the dose levels tested, none of the components caused severe lethargy or lethality. Guinea pigs inoculated intravenously were even more susceptible inasmuch as the addition of as little as 6 µg of MDP to 150 µg of Pseudogen, itself not lethal, caused the death of 80% of the animals. Neither CWS nor P3, in the doses used for tumor treatment, measurably enhanced endotoxic lethality. It has yet to be determined whether the effect described here is related to the known phenomenon of increased susceptibility (hyperreactivity) to endotoxin

observed in mice previously infected with BCG. In any case, our results indicate that caution must be exercised in the application of MDP as a tumor therapeutic ingredient, or as an adjuvant in general, even in combination with relatively weakly endotoxic products, such as Pseudogen, whose antitumor activity is presently being evaluated by others in human patients.

Eight steps were needed for the synthesis of MDP as prepared by French workers in 1975. Dr. Steven Schwartzman shortened the procedure by combining several of the operations and using alanine as a template for the addition of muramic acid and isoglutamine. The efficacy in the line-10 tumor model of several analogues of MDP prepared by this simplified method followed in the order of aminobutyryl-MDP>seryl-MDP > alanyl-MDP. These findings paralleled those obtained in tests for adjuvanticity as measured by the ability of MDP to augment delayed type hypersensitivity to arsenilic tyrosine as well as antibody production to BSA.

Last year we reported that the combination of oil-attached CWS + P3 was found in clinical Phase I-II trials (Dr. Stephen Richman and Dr. Gerald Vosika) to be an effective agent when used intralesionally to treat recurrent melanoma and cutaneous and subcutaneous breast carcinoma. Those studies which were continued during this reporting year have substantiated these results, and a second manuscript describing treatment of recurrent melanoma has been accepted for publication.

Development of effective methods to eliminate tumors remaining after cancer surgery is a major goal of oncologists. Many patients with resectable neoplasmas eventually succumb to progressive growth of tumors originating from occult metastasis present at the time of surgery. Immunotherapy, because it produces specific and systemic antitumor responses is, in theory, capable of eliminating malignant disease remaining after surgery. In collaboration with Dr. Zbar and his co-workers (NCI), we have continued to use the metastasizing line-10 tumor model to help establish conditions for successful post operative immunotherapy. Animals with established dermal tumors were treated either by surgical excision of the tumor or by surgery followed by administration of emulsified cell wall preparations alone or mixed with hepatoma cells. Animals treated by surgery alone developed malignant disease characterized by progressive lethal growth of lymph node metastases. Injection of cell walls or combinations of cell wall components intradermally between the site of excision and the draining lymph node (regional injection) prevented the development of palpable metastasis in some animals. Cell wall components given intradermally on the side contralateral to the surgical site (remote injection) cured no animals. Similar treatments with preparations containing both cell walls and line-10 tumor cells prevented the development of malignant disease in a significant number of animals whether the injections were regional or remote. Treatment with mixtures consisting of living BCG and tumors cells was not as effective as treatment with cell walls mixed with tumor cells. Since injection of living tumor cells with cell wall adjuvant into human patients with minimal residual disease might not be acceptable because of the possibility of tumor growth at the vaccine site, the use of X-radiated

tumor cells was explored. Preliminary data indicate that X-radiated tumor cells admixed with cell walls could replace viable tumor cells in eradicating microscopic lymph node metastases. Whether these findings are relevant to human cancer immunotherapy remains to be seen.

Dr. Werner Brehmer and Dr. Hans Huckauf initiated a Phase I study using CWS + P3 in patients with inoperable carcinoma of the lung. Three to four intratumor instillations of up to 750 µg of CWS and 375 µg of P3 were performed with the aid of a broncoscope in 4 patients and by means of radiologically directed thoracic puncture in 3 patients. Granulomatous reactions were detected in the region of the tumors, but no ill effects were noted. Febrile reactions were either absent or mild with the exception of one case. Since patients tolerated these treatments well, pilot studies were begun in patients with still operable lung carcinoma. The tumors were injected 3 to 4 weeks prior to their surgical removal in the hope that specific tumor immune responses would be enhanced. Of course, a 5-year period is needed to evaluate results of this immunotherapeutic approach.

The major courses of action planned in this project include: 1) expansion of studies with RESI fractions of low toxicity which, in combination with P3, produced excellent regression of animal tumors without noticeable life-threatening effects to determine their chemical and fine structural features responsible for immunotherapeutic properties, 2) attempts to alter MDP so as to minimize its ability to enhance host susceptibility to endotoxin by covalently lipidating it and still retain its tumor regressive properties, and 3) continued collaboration with scientists of other institutes in attempts to identify immunologic cross-reactive components shared by microorganisms and tumor cells and to provide effective antineoplastic agents (microbial components and their synthetic analogs) suitable for clinical evaluation.

Publications:

Richman, S. P., Gutterman, J. U., Hersh, E. M., and Ribi, E. E.: Phase I-II study of intratumor immunotherapy with BCG cell wall skeleton plus P3. Cancer Immunol. Immunother. 5: 41-44, 1978.

Ribi, E., McLaughlin, C. A., Cantrell, J. L., Brehmer, W., Azuma, I., Yamamura, Y., Strain, S. M., Hwang, K. M., and Toubiana, R.: Immunotherapy for tumors with microbial constituents or their synthetic analogues. A review. In Immunotherapy of Human Cancer, 22nd Annual Clinical Conference on Cancer, The University of Texas System Cancer Center, M.D. Anderson Hospital and Tumor Institute, Houston, Texas. New York, Raven Press, 1978, pp. 131-154.

Zbar, B., Canti, G., Ashley, M. P., Rapp, H. J., Hunter, J. T., and Ribi, E.: Eradication by immunization with mycobacterial vaccines and tumor cells of microscopic metastases remaining after surgery. Cancer Res. 39: 1597-1603, 1979.

Publications: (cont'd)

Wepsic, H. T., Tracey, R. S., Harris, S., Ribi, E., and Morris, H.: Bacillus Calmette-Guérin cell wall immunotherapy of intramuscular and metastatic Morris rat hepatomas. Cancer Res. 38: 1217-1222, 1978.

Brehmer, W., Huckauf, H., and Ribi, E.: Immuntherapie mit BCG sowie mykobakteriellen fraktionen und versuche zur anwendung beim bronchial-karzinom. Praxis Pneumonologie 33: 358-365, 1979.

In press:

Vosika, G. J., Schmidtke, J., Goldman, A., Ribi, E., Parker, R., and Gray, G.: Phase I-II study of intralesional immunotherapy with oil attached Mycobacterium smegmatis cell wall skeleton and trehalose dimycolate. Cancer Immunol. Immunother.

Ribi, E. Parker, R., Strain, S. M., Mizuno, Y., Nowotny, A., Von Eschen, K. B., Cantrell, J. L., McLaughlin, C. A., Hwang, K. M., and Goren, M. B.: Peptides as requirement for immunotherapy of the guinea pig line-10 tumor with endotoxins. Cancer Immunol. Immunother.

Vosika, G. J., Schmidtke, J. R., Goldman, A., Ribi, E., Parker, R., and Gray, G. R.: Intralesional immunotherapy of malignant melanoma with Mycobacterium smegmatis cell wall skeleton combined with trehalose dimycolate (P3). Cancer.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00087-02 LMSF

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Immunological Activity of Eukaryotic and Prokaryotic Cellular Components

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. L. Cantrell	Senior Staff Fellow	LMSF NIAID
OTHER:	E. E. Ribi	Res. Chemist (Phys. Chem.)	LMSF NIAID
	C. A. McLaughlin	Senior Staff Fellow	LMSF NIAID
	S. M. Schwartzman	Senior Staff Fellow	LMSF NIAID
	J. Sasaki	Visiting Associate	LMSF NIAID

COOPERATING UNITS (if any) Dr. R. Wheat, Duke University Medical Center, Durham, NC; Dr. G. F. Springer, Evanston Hospital, Evanston, IL; Dr. R. T. Tuttle, Burroughs-Wellcome, Research Triangle Park, NC; Drs. J. Zighelboim and R. I. Murahata, U.C.L.A. School of Medicine, Los Angeles, CA.

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SECTION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	1.0	2.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The main goal of this project is the elucidation of the immunological response induced by isolated microbial components and tumor-associated antigens that are efficacious antitumor agents. In the past year, attempts were made to 1) evaluate fractions of Corynebacterium parvum prepared by phenol-water extraction for lymphoreticular stimulation and antitumor activity, 2) isolate, in soluble form, the component from C. parvum responsible for tumor regression, 3) evaluate inhibition of immunity and induction of suppressor cells with components isolated from microorganisms, 4) evaluate tumor-associated antigens isolated from murine tumor cells and guinea pig ascites fluid for antitumor activity, and 5) determine the cross-reactivity between tumor cells and microbial components.

Project Description:

Many studies have demonstrated that killed suspensions of Corynebacterium parvum possess both lymphoreticular stimulatory and anti-tumor properties. However, other reports show that cell-mediated immune responses attributable to T lymphocytes are often inhibited by systemic administration of C. parvum. Therefore, in collaboration with Dr. R. Wheat, we have continued our efforts to isolate and characterize the component(s) from C. parvum responsible for antitumor activity and/or immune suppression.

In last year's report, preliminary studies demonstrated that both lymphoreticular and antitumor activities were associated with the residue of phenol-water extracted whole cells. Additional studies were made to determine the nature (chemical or physical) of the active component. Fractions were tested in mice for lymphoreticular stimulation by measuring the degree of splenomegaly and spleen cell blastogenesis produced by an i.p. injection. Antitumor activity was evaluated by measuring the extent of tumor growth after a subcutaneous injection of the fractions admixed with either of 3 syngeneically transplanted murine tumors (B-16 melanoma, L1210 leukemia, or MC-93 sarcoma). Significant splenomegaly with concomitant increase in blastogenic activity was observed in mice treated with the residue. The kinetics of development of these parameters paralleled that observed with whole organisms. In addition, significant antitumor activity was observed in mice inoculated with the residue, which was similar to the activity observed with whole cells. The soluble extracts (phenol phase and aqueous phase) did not induce either splenomegaly, increased blastogenesis, or inhibition of tumor growth. High antitumor- and splenomegaly-inducing activities of the residue were retained following chloroform:methanol extraction, SDS/pronase digestion, or RNAse/DNAse/trypsin digestion, whereas these properties were significantly reduced after the residue was treated with metaperiodate, which resulted in a reduction in the carbohydrate concentration. Peritoneal cells from mice treated with whole cells, residue, or metaperiodate-treated residue were nonspecifically cytotoxic to B-16 tumor cells in vitro, but the tumoricidal activity of peritoneal cells did not develop in mice treated with either the aqueous- or phenol-soluble extracts. Collectively, these results demonstrate that the component in C. parvum responsible for antitumor activity and lymphoreticular stimulation is, in part, carbohydrate in nature and that although nonspecific macrophage activation by whole cells or the residue may be important, it is not sufficient for inducing tumor rejection. These findings were reported at the 17th Annual Meeting of the American Association for Cancer Research, May 16-19, in New Orleans, LA.

Studies were also made this year, in collaboration with Drs. J. Zighelboim and R. I. Murahata, into the ability of C. parvum fractions derived by phenol-water extraction to modulate the immune response to tumor alloantigens in mice. When the residue was injected i.v. into mice 9 days after subcutaneous immunization with allogeneic tumor cells, it mimicked the effects resulting from an injection of whole cells. These effects included 1) inhibition of the generation of primary spleen cell cytotoxicity; 2)

inhibition of the generation and expression of memory cytotoxicity by spleen cells; and 3) generation of suppressor cell activity capable of inhibiting the expression of memory cytotoxicity by control alloimmune spleen cells. In contrast, injection of the phenol-soluble extract, which was rich in protein, inhibited the generation of primary and memory cytotoxicity, but did not result in the generation of suppressor cell activity. The aqueous soluble extract, which was rich in carbohydrates, was inactive by all criteria used in this study. These results clearly show that materials extracted from C. parvum differentially effect various aspects of the immune response to alloantigens. These findings were reported at the Leukocyte Culture Conference, May, in Ottawa, Canada.

Studies in this laboratory have also been devoted to isolating, in soluble form, the carbohydrate-like component from C. parvum whole cells that retains the antitumor activity. Heat-killed organisms, supplied by Burroughs-Wellcome, were extracted with pyridine, which is a solvent used to extract glycolipids from mycobacteria, and the soluble extract was tested in vivo for biological and antitumor activities. Local lesional immunotherapy of carcinogen-induced hepatocellular carcinoma (line-10) implanted into the skin of syngeneic guinea pigs was used to measure antitumor activity. Although not significant, tumor regressive activity was detected with the pyridine soluble extract provided that it was combined with trehalose dimycolate (P3) in an oil-in-water emulsion. Based on our previous results that antitumor activity was associated with a cell wall component and the observation that the combination of synthetic muramyl dipeptide (MDP); the smallest adjuvant-active unit of bacterial cell walls; with purified endotoxin, itself devoid of antitumor activity, provided significant line-10 tumor regressive potency (see annual report Z01 AI 00078-07, Ribi), the pyridine-soluble extract of C. parvum was combined with MDP and P3 in an oil emulsion. High cure rates (75 to 88%) were observed in animals given the triple combination, a rate superior to that observed with whole organisms. No activity was seen when the extract was combined with MDP only. In studies designed to evaluate the lymphoreticular stimulating properties of the pyridine extracted fractions, no significant increase in spleen or liver weight was observed in mice treated with the pyridine-soluble extract. Conversely, significant splenomegaly and hepatomegaly were detected in animals treated with the cell residue. Thus, in contrast to our previous results, no correlation could be made between the fraction having antitumor activity and its ability to induce splenomegaly. Additional studies in this laboratory have demonstrated that the inhibition of primary immunity to alloantigens was associated with the splenomegaly-inducing cell residue and not the pyridine-soluble extract. These results suggest that the components of C. parvum responsible for inhibition of immunity and antitumor activity are distinct and separable. Studies are in progress to determine whether the pyridine-soluble extract or residue is involved with the induction of suppressor cell activity.

Another area of interest to us is the use of specific immunostimulants in the regression of established tumors. As reported last year, solubilized tumor antigens prepared by KCl extraction of tumor cells caused complete

regression of EL-4 lymphoma in syngeneic mice. High cure rates (80%) were observed in animals given combination chemotherapy and immunotherapy, rates significantly higher than in chemotherapy only control groups. Successful therapy was dependent on emulsifying the soluble tumor antigen in minute oil droplets. Emulsions containing tumor cell extracts, when tested in conjunction with chemotherapy, had no therapeutic value. Animals cured by combination chemoimmunotherapy had detectable tumor specific immunity. In addition to the murine system, attempts were made to isolate tumor-associated antigens from the ascites fluid of strain 2 guinea pigs with progressively growing line-10 tumors. Butanol-water extracts of the ascites fluid resulted in the acquisition of tumor antigens in the butanol phase. To test the antitumor activity of the butanol phase, intratumor injections of emulsions containing P3 combined with the extract were given to guinea pigs. Antitumor activity (30% cures) was found only in the antigen-containing butanol fraction. We also explored whether the synthetic adjuvant-active MDP would enhance the efficacy of the isolated tumor antigens in regressing line-10 tumors. Preliminary results indicate that high cure rates (88%) were obtained when the isolated tumor antigens were combined with MDP and P3 in an oil emulsion and injected directly into the growing tumor. No activity was seen in animals given this vaccine on the contralateral side.

In collaboration with Dr. G. Springer, studies were undertaken to determine whether line-10 tumor cells and endotoxic fractions isolated from Re mutant of Salmonella typhimurium cross-react with human blood group antigens. Results from these studies indicate that both tumor cells and endotoxins (Re glycolipids and purified B4, see annual report ZO1 AI 00078-07, Ribi) have components that cross-react with T antigen; the precursor to human MN antigens. In addition, tumor cells have membrane components that cross-react with human B antigen. Strain-2 guinea pig cells contain only components that cross-react with human B antigen. Similar findings were observed with the isolated tumor antigens (the butanol extract of ascites fluid). Studies in progress are designed to determine whether purified T antigen is efficacious in regressing established line-10 tumors. Only if this is accomplished will we be able to determine the role of these cross-reactive antigens in tumor regression.

Attempts were also made this year, in collaboration with Dr. Wheat, to isolate the nontoxic, covalently bound MDP-like moieties (Braun's lipoprotein) and pepidoglycan-free endotoxic fractions (Porin) from Re mutant of S. typhimurium, which are outer membrane components, in order to determine their efficacy in regressing line-10 tumors. Results from these studies indicate that the endotoxin-containing fraction, Porin, was ineffective in regressing tumors when administered alone or in combination with P3. Similar results were observed with Braun's lipoprotein. However, significant antitumor activity was observed in animals treated with Porin combined with Braun's lipoprotein. Successful therapy was achieved only upon the proper combination of these components with P3 in an oil emulsion. Antitumor activity was also restored to Porin by the addition of synthetic adjuvant-active MDP. These

results indicate that endotoxicity per se is not sufficient to bring about tumor regression, but when combined with a cell wall adjuvant-like component (MDP or pepidoglycan) antitumor activity is restored.

The future course of this project will include 1) attempts to isolate a homogeneous material from the pyridine-soluble extract of C. parvum by medium pressure silica gel chromatography with the eventual goal of delineation of the structure of the antitumor component, 2) attempts to isolate the component of C. parvum responsible for inducing suppressor cell activation and/or inhibition of allogeneic immunity, 3) attempts to further purify and characterize tumor-associated antigens isolated from guinea pig and murine tumors and to evaluate these antigens for immunoprophylactic and immunotherapeutic value, and 4) continued collaboration with Dr. Springer in attempts to determine whether the cross-reactive antigens shared between line-10 tumor cells and microbial components play a role in tumor regression. Hopefully, results from these studies will enable us to better understand the mechanism of tumor regression and immunosuppression, which will enable us to design immunotherapy protocols that are the most potent and least harmful.

Publications:

Cantrell, J. L., McLaughlin, C. A., and Ribi, E.: Efficacy of tumor cell extracts in immunotherapy of murine EL-4 leukemia. Cancer Res. 39: 1159-1167, 1979.

In press:

Cantrell, J. L. and Wheat, R. W.: Antitumor activity and lymphoreticular stimulation properties of fractions isolated from Corynebacterium parvum. Cancer Res.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00182-01 LMSF

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Biochemical and Genetical Mechanisms of Obligate Intracellular Parasitism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. C. Williams	Senior Asst. Scientist	LMSF NIAID
OTHER:	T. F. McCaul	NIH Visiting Fellow	LMSF NIAID
	M. Peacock	Microbiologist	EB NIAID

COOPERATING UNITS (if any) Drs. E. Weiss and G. Dasch, Naval Medical Research Institute, Bethesda, MD; and Dr. J. Wild, Dept. of Plant Sciences, Genetics Section, Texas A&M University, College Station.

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.75	0.65	1.1

CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this project are to characterize the biochemical and genetical mechanisms of obligate intracellular parasitism between the eukaryotic host and prokaryotic parasite. Molecular interactions at the cell surface and with soluble components are being investigated as primary determinants of metabolic cooperation between host and parasite. Rickettsia typhi utilize host purine nucleotides and in the absence of glutamate, adenosine 5'-triphosphate (ATP) is catabolized to AMP, the end product of ATP catabolism. Therefore, unusual membrane functions contribute to the success of the parasite as a scavenger of critical host components. Metabolic comparisons between autonomously growing bacteria and rickettsiae have revealed exploitable differences in antibiotic sensitivities. A cell wall directed antibiotic has been found which inhibits the growth of R. rickettsii but not Coxiella burnetii or R. typhi. Furthermore, this antibiotic restricts the growth of Rochalimaea quintana and Legionella pneumophila at an ED_{50} of 5 and 12 μg per ml, respectively.

PHS-6040
(Rev. 10-76)

Project Description:

Obligate intracellular parasitism is an enigma which has eluded scientists for several decades. In this laboratory, the nature of the cellular interactions between host and parasite are being investigated at the biochemical and genetical level. Conceptually, metabolic cooperation (MC) between host and parasite is a mechanism of cellular interaction in which the phenotype of the enzyme deficient parasite is alleviated by specified hosts. Studies on MC are designed to elucidate the nutritional requirements of the parasite by applying the rule that these organisms must express certain bacterial functions, whereas key host functions must be preserved. The alleviation of nutritional requirements may be mediated via receptors, informational macromolecules, metabolic intermediates and/or membrane functions.

Investigations into the nature of pyrimidine and purine nucleotide requirements have centered around enzymatic and transport capabilities of purified Rickettsia typhi. Evidence has been presented that these cells require host nucleotides because they do not possess the enzymic requirements for the utilization of bases and nucleosides. Pyrimidine metabolism is greatly restricted by the absence of cytidine 5'-triphosphate (CTP) synthetase which is the only enzyme capable of forming CTP from uridine 5'-triphosphate.

Obligatory features of bacterial functions have been discovered in purine nucleotide metabolism. The metabolism of purine nucleotides by R. typhi is controlled by substrate (i.e., glutamate) availability. ATP is catabolized by whole cells and cell-free extracts of R. typhi to ADP and then to AMP, the end product of ATP catabolism under the experimental conditions which were used. The only intermediate of the pathway from ATP to AMP which was identified by thin layer chromatography and quantitated by the ^{14}C-content was ADP. Products such as adenine, adenosine, hypoxanthine, inosine, IMP, ribose and ribose-5-P were not detected. The enzymes which could be responsible theoretically for the catabolism or anabolism of AMP were not detected by standard assay procedures. Most importantly, 5'-nucleotidase and AMP nucleosidase activities were undetectable under a variety of experimental conditions. Although these two enzymes in other cells remove AMP from the adenylate pool, they are apparently nonfunctional with R. typhi since the adenylate energy charge was drastically lowered by the high proportion of AMP. The biosynthesis of ATP was initiated by adenylate kinase because no adenine phosphoribosyltransferase or adenosine kinase could be detected. Hence, the salvage of purine bases and nucleosides appears to be nonfunctional, at least, in R. typhi. Adenylate kinase was readily separated from GMP kinase by column chromatography and sedimentation in sucrose gradients, whereas no IMP kinase activity was detected. Although no IMP formation was detected with ^{14}C-AMP as substrate, previous analyses of endogenous nucleotide pools by high pressure liquid chromatography indicated that IMP exists in R. typhi. The phosphorylation of nucleoside diphosphates is carried out by a single enzyme with a pI of 5.0. These observations suggest that the in vivo activity of adenine nucleotide interconversion is limited to the nucleotides with AMP being the end product of ATP catabolism and that the salvage of purine bases and

nucleosides is not an essential feature of purine metabolism. Future studies are designed to determine the in vivo molecular interactions between rickettsial membrane function and host-derived components.

Metabolic comparisons between gram-negative rickettsiae and autonomous bacteria have, until recently, provided little evidence that primary differences could be exploited in terms of antibiotic sensitivity. Use has been made of the cell wall directed antibiotic fosfomycin (FFM) which enters cells via the hexose phosphate (uhp) and the L-α-glycerophosphate (glt) transport system. Three strains of the genus Rickettsia were tested for susceptibility to FFM by their growth in tissue culture, embryonated hens' eggs and/or in mice. The drug was not cytotoxic to embryonated eggs at 10 mg per egg, mice at 0.6 mg per mouse or in tissue culture LM3 cells at 0.1 mg per ml. Observations with R. typhi grown in irradiated or unirradiated LM3 cells indicated that FFM may have a growth inhibitory action at concentrations that were cytotoxic for the host cells. The biological activity (ability to hemolyze sheep red blood cells) of R. typhi was not impaired at concentrations of 33 µg per ml. In embryonated eggs 10 mg per egg did not alter the average day of death (ADD) of the embryos. In mice, FFM at 0.6 mg per mouse did not alter the ADD of mice injected with a sufficient dose of R. typhi to kill 100% of the animals within 1 week. Coxiella burnetii was resistant to FFM up to 10 mg per egg (250 µg/ml), whereas R. rickettsii was sensitive. Epicellular organisms such as Rochalimaea quintana and Legionella pneumophila were sensitive to the effects of FFM at an ED_{50} of 5 µg per ml and 12 µg per ml, respectively. Embryonated eggs were easily eradicated of L. pneumophila at 5 mg per egg. FFM can be employed during the infection cycle and growth phase in the presence of the drug since R. typhi and C. burnetii are not susceptible. Therefore, there appears to be no direct antibiotic effect on extracellular rickettsiae which have not penetrated cells and started to multiply. More importantly, it is not necessary to remove the antibiotic for the infection to proceed from cell-to-cell. Since FFM is bacteriocidal, it allows the complete elimination of epicellular organisms and autonomously growing contaminants. The mechanism of this apparent resistance of rickettsiae to FFM is currently under investigation. These studies will provide information on cell wall biosynthesis and the molecular interactions between rickettsial and host determinants.

Investigations carried out on the rickettsiae require that an autonomously growing gram-negative bacterium be used whenever possible as a positive and negative control. We have routinely used Salmonella typhimurium LT2 and various strains to demonstrate enzymatic activity and transport functions. If an enzyme or transport process is undetectable in the rickettsiae, then appropriate mixing of rickettsial extracts to fractions of S. typhimurium must be carried out so that a determination can be made about specific or nonspecific enzyme or transport inhibition of rickettsiae. Previous studies with R. typhi indicated that the transport of uridine did not occur; however, S. typhimurium transport was clearly demonstrable.

S. typhimurium LT2 possesses complex transport systems for uracil and uridine which have been analyzed in mutations affecting pyrimidine salvage of bases and nucleosides. The transport of intact uridine is characterized by an apparent Km of 4.28 ± 0.37 μM with an apparent Vmax of 5.35 ± 0.18 pmole min^{-1} μg $protein^{-1}$. Uridine transport requires a binding protein, an enzymatic component (uridine kinase, URKase encoded by udk), and is subject to induction by uridine. The transport of uracil depends upon a binding protein and an enzymatic component (uracil phosphoribosyltransferase, UPRTase encoded by upp). Four mutants which are defective in components for base and nucleoside salvage were employed to characterize the transport systems according to 1) their transport of uracil and uridine, 2) the presence or absence of specific enzymes, and 3) the localization of the enzymes and binding proteins. Strains S177 and HD1043 are $UPRTase^-$ and do not transport uracil, whereas strain JL411 ($URKase^-$) transports uracil at 89% and uridine at 5% of the rate of strain LT2. Periplasmic binding proteins were identified as components of both transport systems, and the uridine binding protein was identified as a separate component from URKase. A schematic model for the transport of uracil, uridine and uridine from uridine 5'-monophosphate is presented in which the first step involves the participation of a periplasmic binding protein for uracil or uridine. Uridine may be degraded to uracil and ribose-1-P, whereas uridine 5'-monophosphate is degraded to uridine and inorganic phosphate with subsequent binding of the pyrimidine moiety. The transport system is highly discriminatory reflecting the components of the model from which a new class of mutants may be defined as those cells lacking uracil or uridine binding activity.

Since R. typhi did not transport uracil or uridine, we tested the transport of pyrimidine nucleotides. Preliminary results indicate that the nucleotides are transported intact by R. typhi. This is clearly a property of obligate intracellular parasitism, and more studies are required to determine the complex nature of their transport system.

The future course of this project will center around 4 areas as specified below: 1) The in vivo molecular interactions between rickettsial membrane function and host-derived components will be investigated by ultrastructural cytochemistry and autoradiography. The objective is to show metabolic cooperation between host and parasite during the infection cycle by observing the expression of bacterial functions. Cell organelle and membrane(s) will be examined for phosphatase activities which are absent in the parasite but present in the host. 2) Utilization of host nucleotide pools by R. typhi in selected tissue culture strains will be examined. An attempt will be made to correlate rickettsial growth with nucleotide availability. The bacterial enzymes participating in nucleotide pool interconversions will be analyzed. 3) Cell wall biosynthesis by C. burnetii, R. typhi and R. rickettsii will be studied. The differential sensitivity to FFM already demonstrated will be used as a probe. 4) Rickettsial and legionaires strains will be screened for plasmids. If plasmids are found, functional

aspects of parasitism may be correlated with protein patterns, metabolic differences, and such characteristics as enhanced or depreciated virulence, invasiveness, etc.

Publications:

Williams, J. C. and Weiss, E.: Energy metabolism of Rickettsia typhi: pools of adenine nucleotides and energy charge in the presence and absence of glutamate. J. Bacteriol. 134: 884-892, 1978.

Williams, J. C., Kizaki, H., Weiss, E., and Weber, G.: Improved radioisotopic assay for cytidine 5'-triphosphate synthetase (EC 6.3.4.2) Anal. Biochem. 91: 46-59, 1978.

In press:

Williams, J. C., Lee, C. E., and Wild, J. R.: Genetic and biochemical characterization of distinct transport systems for uracil, uridine and cytidine in Salmonella typhimurium. Mol. Gen. Genet.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00183-01 LMSF

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Structural and Functional Relationships of Bacterial Antigens in the Immune Response

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. C. Williams	Senior Asst. Scientist	LMSF NIAID
OTHER:	E. E. Ribi	Res. Chemist (Phys. Chem.)	LMSF NIAID
	J. L. Cantrell	Senior Staff Fellow	LMSF NIAID
	T. F. McCaul	NIH Visiting Fellow	LMSF NIAID
	M. Peacock	Microbiologist	EB NIAID

COOPERATING UNITS (if any) Drs. E. Weiss and G. Dasch, Naval Medical Research Institute, Bethesda, MD and Dr. C. O. Kindmark, Asst. Head of the Dept. of Infectious Diseases, University Hospital, S-75014 Uppsala, Sweden

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.20	1.95	1.25

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this project are to characterize the antigens of the rickettsiae and other bacteria which are pathogenic for humans. Structure and functional aspects of the immunomodulation by the cell wall and soluble components are currently being analyzed as potential candidates for subunit vaccines and as tumor-regressive components. Studies employing Coxiella burnetii have revealed a particulate component which is nontoxic, provides protection against a lethal challenge in mice and guinea pigs and regresses the line-10 tumors in strain-2 guinea pigs. This particulate material is potentially a nontoxic vaccine. Soluble components have been identified as putative early diagnostic antigens which induce specific antibody during infection but not after immunization.

PHS-6040
(Rev. 10-76)

Project Description:

The objectives of this project are to elucidate the chemical structure required for the activation of the immune responses against bacterial infections. Specifically, our goal is the establishment of standard preparative procedures for fractionating whole cells into constituent components and the analysis of each component in animal models and in vitro studies. Fractionation procedures involve direct whole cell extractions with organic solvents, separation of the cell wall components and analysis of aqueous-soluble fractions. The constituent components of each fraction will be evaluated for immunological efficacy in the mouse, guinea pig and rabbit. The ultimate aim is the development of standard procedures for rapid analysis of various components of microorganisms pathogenic for humans. Subsequently the establishment of such procedures will lead to basic studies on the development of limited use vaccines.

A new procedure has been devised for the purification of live and infectious Coxiella burnetii in gram quantities from the yolk sacs of embryonated eggs. This technique allows purification to homogeneity and complete separation from host components. Organisms purified by this procedure are being employed for biochemical, electron microscopic and immunological studies.

Current investigations in collaboration with Drs. Cantrell and Ribi and Mr. Peacock are being conducted with C. burnetii. Studies designed to evaluate the biological activities and immunoprophylactic capabilities of whole cells and/or cellular components have revealed the following results:

1) Killed whole cells of C. burnetii induce severe splenomegaly, liver necrosis, and blastogenic activity in mice. The severity of these phenomena was dose dependent. In fact, high doses (300 µg per mouse) of killed whole cells were lethal for C57BL/10 and not for BDF_1 (C57BL/6 X DBA_2). The live organism was lethal for C57BL/10 with an LD_{50} of 2.6 X 10^8 organisms per mouse.

2) The residual particulate material from chloroform:methanol (C:M)- or hot phenol:water-extracted whole cells did not induce severe splenomegaly, however lymphocyte stimulating activity was retained. More importantly, no liver necrosis was detected in mice given 1 mg of the C:M residue.

3) Humoral immunity, as measured by microagglutinin and fluorescent antibody techniques, was induced in mice immunized i.p. with either whole cells or C:M residue. The antibody titer in sera from mice given C:M residue was decreased following absorption with either whole cells or C:M residue. A similar reduction in antibody titer was observed in the sera of mice immunized with whole cells after absorption with C:M residue. No decrease in antibody titer was seen after these sera were absorbed with the aqueous phenol:water extract.

4) Immunodiffusion studies detected at least 2 different antigens in the proxoplasm of C. burnetii which were not identical to those observed in the aqueous phenol:water and C:M soluble extracts. At least one additional antigen has been detected as being related to infection, whereas immunization with killed whole cells or with fractions does not induce an antibody response to this antigen. Soluble antigens from live preparations have been identified which were not detected when formalin was used to kill the organisms before purification.

5) Studies using the guinea pig have revealed that C. burnetii are lethal at high concentrations, whereas killed whole cells are not lethal. The C:M residue induces humoral antibody and protects against a lethal challenge (10^{10} organisms per guinea pig).

6) Anti-tumor activity in suppression tests was observed in mice given tumor cells admixed with either whole cells, cell walls, or purified whole cells of C. burnetii. Conversely, no anti-tumor activity was seen in tumor cells treated with aqueous phenol:water extract or C:M residue. Regression of the line-10 tumor in the strain-2 guinea pig was observed with whole cells, cell walls and the C:M residue. The C:M residue from extracted whole cells and cell walls appear to be a good candidate for a nontoxic vaccine since the present whole cell vaccine induces all of the severe side effects.

In collaboration with Dr. C. O. Kindmark, soluble protoplasmic components are being investigated for the rapid diagnosis of rickettsial diseases. Currently we are studying a soluble antigen from C. burnetii which is obviously recognized by the immune system during infection but not during immunization. The detection of circulating antibodies and/or antigen in patients will be useful in the clinical management of Q fever.

The future course of this project will center around the 3 areas as specified below: 1) C. burnetii will be fractionated into cell wall and protoplasm. The protective antigens will be localized and separated from toxic factors and the efficacy of each component will be compared to the present whole cell vaccine. 2) Membrane proteins, carbohydrates and other components will be compared in various strains of rickettsiae. Extracellular proteins and glycoproteins will be identified by ^{125}I labeling patterns and two-dimensional chromatography. Carbohydrates will be identified by cytochemical staining. 3) Early detection of antigens and antibodies will be attempted by employing homogeneous antigens and monospecific antisera. Antibody classes and subclasses induced by various homogeneous antigens will be analyzed during immunization as compared to infection carried out in mice and guinea pigs.

Publications: None

Laboratory of Persistent Viral Diseases
Rocky Mountain Laboratories
Hamilton, Montana
1979 Annual Report
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Annual Report
Laboratory of Persistent Viral Diseases
Rocky Mountain Laboratories
Hamilton, Montana
National Institute of Allergy and Infectious Diseases
October 1, 1978 to September 30, 1979

RESEARCH HIGHLIGHTS

The influence of the Rfv-1 and Rfv-3 genes on Friend virus leukemia. The Rfv-1 gene, associated with the H-2D region of the major histocompatibility complex, has a strong influence on recovery from Friend virus-induced leukemia. Recent studies using irradiation chimeras indicated that the Rfv-1 effect could be transferred by spleen and bone marrow cells to irradiated recipients. Furthermore, transfer of spleen cells from neonatally tolerized mice to nonirradiated H-2 hemi-syngeneic mice suggested that the Rfv-1 (H-2D) genotype of the leukemia cells did not determine the Rfv-1 effect on recovery from leukemia. On the contrary, the Rfv-1 genotype of the immune system appeared to control the incidence of recovery from leukemia. Our current interpretation is that the Rfv-1 gene influences the kinetics of the virus-specific cytotoxic T lymphocyte response to FV leukemia cells, and thus appears similar to other H-2D-associated immune response genes which influence cytotoxic T lymphocyte responses.

The Rfv-3 gene acts in a complementary manner with Rfv-1 to influence recovery from leukemia. The exact linkage of Rfv-3 is not known, but it is not linked to the H-2, Fv-2 or Ig-1 loci. Rfv-3 may be an "immune response gene", as the Rfv-3$^{r/s}$ genotype is associated with a positive cytotoxic anti-FV leukemia cell antibody response. This occurs also in the face of persistent leukemia in association with the low recovery Rfv-1 genotype. These anti-FV antibodies cause modulation of virus-induced leukemia cell surface antigens. Leukemic spleen cells become insensitive to lysis by antibody and complement and show a dramatic reduction in virus release in spite of persistent infection. As a result, cell-free spleen virus titers are decreased and viremia is eliminated. It is currently unclear whether the modulation of FV cell surface antigens interferes with elimination of leukemia cells by cytotoxic T lymphocytes. However, the genetic complementation observed between the Rfv-1 and Rfv-3 genes which is necessary for recovery from leukemia would suggest that these effects are not antagonistic and may involve different viral cell surface molecules. (Britt, Chesebro, Doig)

Variations in Friend virus-expression in leukemia cell clones. FV leukemia cell clones with variations in expression of virus-induced cell surface proteins have been isolated. One clone is deficient in processing of "gag" proteins (p10, p12, p15, p30) but has normal "env" proteins (gp70

and p15E). Another clone has normal "gag" expression but lacks the "env" proteins. These clones are being used to characterize the specificity of host humoral and cell-mediated immune responses to FV leukemia. In addition, they are being studied as models of non-virus producing persistently infected cells which appear late in the course of leukemia in mice with the Rfv-$3^{r/s}$ genotype. (Collins, Chesebro)

Alteration of expression of H-2K and H-2D region molecules in a clone of virus-induced leukemia cells. A clone of leukemia cells derived from an H-$2^{b/b}$ lymphoma (FBL-3) was found to be devoid of expression of antigens determined by the H-2D subregion. On the other hand, H-2K determinants recognized by serological reagents were normally expressed. However, cells of this clone were unable to stimulate or block T cell-mediated cytolytic responses directed at H-$2K^b$ determinants. The dissociation of antibody-mediated and T cell-mediated recognition of H-$2K^b$ determinants in a homogenous cell population implies that unique determinants may be recognized by each of these immune mechanisms. The lack of any H-$2D^b$ determinants on these cells suggests a possible role of H-$2D^b$ region in control of some aspects of H-$2K^b$ recognition or expression. These findings are being pursued by biochemical analysis of the H-$2K^b$ molecules from this clone compared to molecules from normal spleen cells and EL-4 lymphoma cells. Additional variant cell lines are also being selected in the presence of specific antibody or cytotoxic T cells to study the relationship between H-2K and H-2D subregions. [Portis, Kindt (Laboratory of Immunogenetics), Colligan (Laboratory of Immunogenetics)]

M. tuberculosis vaccine induces a unique type II interferon in mice. Mice inoculated with an oil droplet emulsion of nonviable M. tuberculosis exhibit enhanced resistance to encephalomyocarditis virus (EMCV). This effect is not virus-specific since vaccinated mice also show increased resistance to herpes simplex virus (type II) and rabies virus. Recent experiments indicate that a soluble antiviral mediator can be found in tissue culture fluids from peritoneal cells removed from vaccinated mice. This factor blocks the spread of EMCV and other virus infections in cell monolayers in vitro. This mediator is neutralized by antiserum to mouse type II interferon, but differs from type II interferon in that it is acid (pH 2) sensitive and heat labile, and is inactive in a subline of mouse L cells in which other preparations of mouse type II interferon are fully active. (Lodmell, Pusateri, Cent)

Characterization of Aleutian disease virus of mink. The Gorham strain of Aleutian disease virus (ADV) of mink has been grown in Crandall feline kidney cells, labeled with ^{3}H-thymidine and purified in CsCl gradients. Separate peaks of virus infectivity were observed at densities of 1.35 and 1.43 gm/ml. These densities and the incorporation of thymidine into virion nucleic acid suggests that these viruses are both members of the parvovirus group. Although neither virus peak produced virulent infection in sapphire mink, both cross-reacted antigenically with a virulent virus strain (Utah I) purified from mink with Aleutian disease. (Bloom)

Cell-mediated immune response to Aleutian disease virus of mink. Certain strains of mink infected with Aleutian disease virus (ADV) develop very strong specific antiviral antibody responses and usually succumb to immune complex glomerulonephritis or arteritis. Lack of successful regulation of the immune response to ADV appears to be a significant factor in this disease since serum immunoglobulin levels are often elevated to levels as high as 50 mg/ml and many organs become massively infiltrated with plasma cells. To study cellular aspects of immunoregulation during Aleutian disease, an ADV-specific T lymphocyte proliferative assay has been developed. Mink with advanced disease have strong responses, and those in early stages of disease have weak responses. The cellular immune response to ADV is being compared to the response to protein antigens (KLH and HGG) to search for antigen-specific suppressor cells capable of regulating the immune response to these antigens and see if such cells are absent in ADV-infected mink. (Race, Coe)

Structural and genetic analysis of hamster female protein (FP). A sex-limited serum protein (FP) of female hamsters has been found to exist in electrophoretically distinct, but antigenically similar, forms in 3 inbred strains of hamsters. Using these electrophoretic variants, genetic studies are now under way to search for possible linkage of FP to the major histocompatibility locus in hamsters because of the similarity between FP in hamsters and SLP in mice. (Coe)

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ADMINISTRATIVE REPORT

Administrative, organization and other changes. The Laboratory of Persistent Viral Diseases was created during the past year and has drawn together in a single group the researchers at the Rocky Mountain Laboratories working on various aspects of virus-host interactions in persistent virus diseases.

During the past year 3 graduate students from the Department of Microbiology, University of Montana, Mr. R. Cent, Ms. A. Pusateri and Mr. W. Tino, were appointed student workers to carry out the research portion of their graduate studies under the preceptorship of members of the laboratory. Mr. D. Doig, Department of Microbiology, Montana State University, left the laboratory after completion of research for the Ph.D. degree under Dr. Chesebro.

Dr. Frank Waxman and Dr. Miles Cloyd joined the laboratory in August to begin work as Staff Fellows under Drs. Coe and Chesebro, respectively.

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HONORS AND AWARDS

Editorial Boards of Journals:

Dr. B. Chesebro

Associate Editor - Journal of Immunology
Ad hoc Reviewer - Journal of the National Cancer Institute
- Proceedings of the National Academy of Sciences

Dr. D. L. Lodmell

Associate Editor - Journal of Immunology

Professional Posts:

Dr. B. Chesebro - Member, Immunobiology Study Section, Division of Research Grants, NIH; Adjunct Professor - Department of Microbiology, Montana State University, Bozeman.

Dr. D. L. Lodmell - Faculty Affiliate, Department of Microbiology, University of Montana, Missoula

Invited Lectures and Participation in Meetings and Symposia:

Dr. B. Chesebro - Invited Speaker, Gordon Research Conference on Viruses and Animal Cells, Boston

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00072-08 LPVD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Host Defense Mechanisms in Viral Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. L. Lodmell	Senior Scientist	LPVD NIAID
OTHER:	J. L. Portis	Medical Officer (SURG)	LPVD NIAID

COOPERATING UNITS (if any)
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major objective of this project is to delineate, at the humoral and cellular level, the mechanisms of host resistance to viral diseases. The principal model for these studies involves induction of nonspecific resistance of mice to encephalomyocarditis virus infection by an oil-droplet emulsion of nonviable Mycobacterium tuberculosis. Additional studies have been initiated to study the pathogenesis and neuroimmunology of rabies virus-infected mice, and to ascertain the mechanism(s) by which mice abort central nervous system infections and recover from paralytic disease.

PHS-6040
(Rev. 10-76)

Project Description:

Nonspecific resistance to encephalomyocarditis virus infection. Previous studies focused on an in vivo model to investigate the humoral and cellular immune functions of mice that exhibit enhanced resistance to a lethal challenge of encephalomyocarditis virus (EMCV) following administration of an oil-droplet emulsion of nonviable Mycobacterium tuberculosis. Similar concentrations of virus in plasma of normal and mycobacteria-sensitized mice from 1 to 120 minutes after EMCV challenge indicated that resistance was not a result of rapid elimination of virus from the circulation, and survival of viremic mice showed that protective mechanisms were operative after EMCV had replicated. Additional studies determined that neonatal thymectomy had no effect on protection, but that splenectomy after mice had been sensitized with mycobacteria, or the intraperitoneal injection of silica 24 hours before virus challenge abrogated resistance to EMCV.

As a continuation of this work, we have focused our efforts on determining the mechanisms of this nonspecific resistance in an in vitro assay system. In collaboration with Anne Pusateri, (Master's Degree Candidate, Department of Microbiology, University of Montana, Missoula), it has been confirmed that peritoneal cells (PC) from sensitized mice, as compared to PC from normal animals, inhibit EMCV replication in mouse embryo fibroblast monolayers by ≥ 2 $\log_{10}$. PC from mice that received mycobacteria either intravenously or intraperitoneally (i.p.) inhibit replication equally well at an effector to target ratio of 20; at lesser concentrations PC from i.p. sensitized animals were more effective. PC collected 2 to 6 weeks after sensitization were the most effective in inhibiting replication (>99% inhibition), but marked inhibition (>90%) also was detected with PC harvested at 7 to 10 weeks. These results correlate with the time frame of our previous in vivo protection studies.

Kinetic studies to determine how rapidly PC from sensitized mice exert their inhibitory effect showed that minimal inhibition (50%) occurred 8 hours post-addition of PC to monolayers and that 90% inhibition occurred if PC were in contact with monolayers 10 hours. Thereafter, virus titers in monolayers incubated without PC or normal PC continued to increase, whereas viral replication in monolayers incubated with PC from sensitized mice was abrogated (>99% inhibition). It also was determined that PC added to monolayers as late as 8 hours after infection inhibited replication 90%.

The release of similar amounts of ^{51}Cr from uninfected monolayers incubated with media or PC from sensitized or normal mice indicated that PC did not kill target cells. Furthermore, similar viral titers in supernatant fluids removed from macrophage cultures prepared from PC of sensitized and normal mice, as well as media without cells, showed elimination and/or inactivation of infectious EMCV by macrophages were not important in this system. It also was determined that macrophages from neither normal nor

sensitized mice supported EMCV replication. Additional studies showed that nonviable PC failed to inhibit viral replication and that factors such as pH and depletion of culture nutrients were unimportant.

At this point, an antiviral mediator was considered to be responsible for inhibition of viral replication because 1) Marked inhibition (≥90%) was not detected unless PC were in contact with infected monolayers for 8 to 10 hours. 2) Cytotoxicity was not detected in monolayers incubated with PC. 3) EMCV was not inactivated and/or eliminated by macrophages. 4) PC did not inhibit viral replication if every cell in the monolayer initially was infected. After an extensive and exhaustive search, an antiviral mediator was detected in supernatant fluids of PC cultures from sensitized but not normal mice. The mediator was not present in supernatant fluids harvested 2 hours postincubation, whereas media harvested at 4 hours had slight activity and media harvested ≥8 hours markedly inhibited replication (>90% to >99%).

The mediator has been characterized as a Type II interferon in that it is neutralized by anti-Type II interferon but not anti-Type I interferon. However, it is a unique Type II interferon in that 1) It is labile to either treatment at pH 2.0 for 24 hours at 4°C, or heat at 56°C for 1/2 hour. 2) It must be in contact with monolayers in an interferon assay for a minimum of 18 hours to inhibit VSV replication, whereas Type I and Type II interferons inhibit replication after 2 and 8 hour incubations, respectively. 3) It is inactive on the mouse L cell routinely used in our laboratory, whereas two different mouse Type I and Type II interferons tested at similar units/ml express activity on this same cell.

The effector cell from the peritoneal cavity of sensitized mice responsible for inhibiting EMCV replication is presently being determined (Master's Degree project, Robert R. Cent, Jr., Department of Microbiology, University of Montana, Missoula). Preliminary results indicate that the cell adheres to plastic, nylon wool and baby hamster kidney microexudates, but is not phagocytic. The classical T cell does not appear to be the effector cell because PC from sensitized athymic nude mice inhibit EMCV replication more effectively [greater inhibition at lower effector-target ratios] than PC from their euthymic littermates or the C57BL/10ScN mice used in our assays.

The future course of the project associated with induction of nonspecific immunity with nonviable mycobacteria will focus on identification of the effector cell(s) responsible for inhibition of EMCV replication. In addition, a more precise characterization of the unique Type II interferon that is associated with protection will be undertaken and a determination made as to whether the effector cell produces this Type II interferon or whether cell cooperation is needed.

Host defenses in rabies virus infection. Studies initiated to study the pathogenesis and neuroimmunology of rabies virus-infected mice, and to ascertain the mechanisms by which mice abort central nervous system (CNS) infections and recover from paralytic disease are in the developmental stage. The technique for harvesting cerebral spinal fluid (CSF) from mice has been mastered. Using this technique, it was shown that high titered interferon present in blood is not detectable in the CSF. These results indicate that if antiviral mediators are present in the CNS and do correlate with abortive infections, they probably are produced locally. It also has been determined that the occurrence of abortive infections in either C57BL/10ScN or RML mice is not increased if different strains of street virus isolated from bats, bobcat or fox are used for infection; abortive infections are still most readily induced, as has been previously established, by the intraperitoneal inoculation of 18 to 21 day old mice with low passage street viruses. We have, however, induced occasional abortive infections in mice following intramuscular (i.m.) inoculation of street and fixed viruses. This observation is contrary to accepted dogma, and indicates that an experimental model for abortive infections following the natural route of infection (i.m. inoculation) may be adaptable for further investigation.

The rabies virus project will focus on the induction of abortive infections in various strains of mice following i.p. inoculation with virulent street virus, and the use of an attenuated rabies virus vaccine to induce abortive infections after intracerebral inoculation. With the attenuated rabies virus vaccine model, >75% of the mice become sick, abort infection and survive. Thus, mechanisms of survival can be more readily studied and the results applied to subsequent investigations with virulent street viruses. Special emphasis will be placed on detection and characterization of antiviral mediators and antibody present in the CSF of mice that have aborted infections.

Publications:

Lodmell, D. L. and Ewalt, L. C.: Induction of enhanced resistance against encephalomyocarditis virus infection of mice by nonviable Mycobacterium tuberculosis: mechanisms of protection. Infect. Immun. 22: 740-745, 1978.

In press:

Lodmell, D. L., Cent, R. R., Jr., Pusateri, A. M. and Ewalt, L. C.: Nonspecific inhibition of encephalomyocarditis virus (EMCV) replication by a nonphagocytic adherent cell from athymic nude mice sensitized with nonviable Mycobacterium tuberculosis. Proceedings Third International Workshop on Nude Mice.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00073-14 LPVD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Mechanisms of Immunity and Immunopathology in Virus Related Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. E. Coe	Medical Officer	LPVD NIAID
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	M. E. Bloom	Medical Officer (Res.)	LPVD NIAID
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NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	1.5	2.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Our studies have been oriented to define the role of Ig classes in the immune response/pathophysiology in viral diseases. The immune complexes of Aleutian diseases (AD) mink are being analyzed to explain the predominant deposition of IgA in glomerulae of terminal AD mink. Also, the aberrant lymphoproliferation in AD mink is being studied by analyzing T cell mitogen responses to protein and AD virus antigens. SV-40 inoculated into Syrian hamsters has been found to induce B cell lymphomas which are preferentially of an IgG class. Some of these tumors also secrete IgG which is monoclonal and the product varies from whole Ig molecules with free L chains to primarily free H chains. In contrast, IgM synthesis was preferentially enhanced by injection of Freund's adjuvant combinations into Syrian hamsters. The essential mycobacterial component of Freund's adjuvant is present in the cell wall but is not MDP.

PHS-6040
(Rev. 10-76)

Project Description:

Studies on the aberrant lymphoproliferative response in Aleutian diseased mink. Aleutian disease (AD) of mink is a persistent virus infection in which viremia is associated with circulating antibody. Infected mink usually die of glomerulonephritis and/or arteritis which is probably related to the deposition of immune complexes.

Of particular interest is the high, apparently uncontrolled, immunoglobulin synthesis that occurs in Aleutian disease. Serum immunoglobulin levels often reach 50% or more of the total serum protein component (i.e., ~50 mg/ml), and increase steadily in amount as the disease progresses.

Our studies are designed to elucidate the cellular mechanisms that may be important in regulating immunoglobulin synthesis and identifying cellular subtypes responsible for the lack of immune regulation in AD. In addition, we intend to identify viral antigens or viral induced antigens that may be the initiator and/or target of the immunoglobulin synthesis.

In order to study cellular immunity in mink, a lymphocyte blastogenesis assay has been developed and adapted to mink lymph node populations. Optimum conditions have been defined and the assay utilized to observe cellular responsiveness of lymph node cells to a variety of nonspecific mitogens as well as specific antigens with which mink have been sensitized [keyhole limpet hemocyanin (KLH), human gamma globulin (HGG)] and also preparations containing Aleutian disease virus (ADV).

Definitive proliferative responses (3- to 8-fold over background), have been found with specific antigens. Comparison of various lymph nodes from individual animals has shown some variation of response. For example, within individual mink, mesenteric nodes were frequently more reactive than peripheral lymph nodes to KLH. Sequential observation of the response in individuals by lymph node biopsy, reveals no decline in the magnitude of response over at least a 4 month period but some lymph node to lymph node variation. Lymphocytes from mink with advanced Aleutian disease had a strong blastogenic response (mean increase over background of 11) to ADV containing preparations but not to appropriate (non-ADV) controls. Smaller responses (2-fold) were detected in lymphocytes from animals less severely affected or shortly after infection. Specific responses were markedly enhanced by purifying the "T" cell population; cells which passed through nylon wool columns were morphologically (absence of membrane bound Ig) and physiologically (response to concanavalin A) T cells. These cells had low background counts and were the major responders to KLH, HGG, and ADV.

Immune complexes (IC) are the hallmark of AD, and previous work in this laboratory has shown a preferential deposition of IgA in AD mink glomerulae. Although serum IgA shows dramatic increases in AD affected mink, other classes (IgG) almost certainly must contribute to the circulating IC. The selective presence of IgA in glomeruli suggests that circulating IgA-IC

are preferentially deposited or selectively preserved. One approach to this problem is to define the Ig classes represented in the putative circulating IC of AD and the specificity of the antibody (i.e., antigens responsible for IC). The objective of current work is to isolate and analyze the components of the immune complexes. Qualitative and preparative techniques we are developing in this regard include: 1) The Raji cell radioimmune assay which quantifies complexes binding to complement receptors on the lymphoblastoid cells, 2) Gel filtration followed by affinity chromatography in which high molecular weight serum components containing putative complexes are bound by staphylococcal-protein A affinity chromatography, and 3) Ultracentrifugation.

The assays developed or being developed appear to offer a reliable means for studying and defining the sequential cellular and humoral events that occur during the pathogenesis of Aleutian disease in susceptible and resistant mink.

Characteristics of SV-40 induced hamster lymphomas. Previous studies in this laboratory have shown that lymphoma induced by SV-40 inoculation of young hamsters were composed of B cells with membrane Ig (MIg) detectable by fluorescent microscopy. Of particular interest was the predominance of a 7S Ig (IgG_2) in 4 of 5 different tumors examined. Only 1 of 5 tumors had surface IgM in contrast to mouse lymphomas which characteristically have IgM or IgD H chains expressed on the surface. These SV-40 B cell lymphomas have been evaluated for their secretory products, i.e., whether the cell secretes Ig which is identical to MIg and is structurally comparable to serum Ig. Analysis of ^{35}S methionine cultured cells by specific precipitation followed by SDS-PAGE, has shown 3 of 4 tumors with IgG_2-MIg are secretors. However, their products differ as one tumor is producing a large excess of L chains ($\sim$40,000 daltons, presumably a dimer) in addition to whole IgG_2 (160,000 daltons) and another tumor appears to produce only H chain dimers (found predominantly intracellularly). Another SV-40 tumor (the only non-IgG_2 MIg which is faintly positive for IgA-MIg) secretes a Ig molecule with a $\simeq$70 K dalton H chain which reacts best with nonspecific (anti-L chain) antisera and may represent hamster IgD. Several other non-SV-40 hamster lymphomas have been defined, for example, a plasmacytoma originally described by Fortner, has been shown to secrete IgA, although the H chain is consistently larger (70 to 100 K daltons) than the IgA H chain synthesized by normal hamster mesenteric lymph node (60 to 65 K daltons). This large IgA H chain does not appear to be a carbohydrate artifact of SDS-PAGE but may represent a molecule with a persistently attached "secretory" component which should be split off by a glycosidase. Further work will identify the probably monoclonality of these SV-40 induced tumors and attempt to clarify the relationship between a SV-40 receptor and IgG_2-MIg, as IgG B cell lymphomas are of especial rarity in other model systems (mouse) and in man. Also, the mechanism and specificity of tumor immunity, which has been observed in appropriately inoculated hamsters, will be investigated.

Selective stimulation of IgM synthesis by Freund's adjuvant. Freund's adjuvants have a well known effect to markedly amplify the immune response to antigens contained in the emulsion. We have found that Freund's adjuvants also have a profound effect on IgM synthesis in the Syrian hamster under certain circumstances. Injection of incomplete Freund's (IF) adjuvant intraperitoneally (i.p.) and complete Freund's (CF) adjuvant in footpads (FP) will induce a profound increase in serum IgM levels (values from 20 to 50 mg/ml are frequently observed vis normal levels of ≈1.0 mg/ml. This particular combination is necessary as the emulsion administered individually, or at opposite sites produce only modest (4 to 8 mg/ml) increases. Male hamsters average higher levels (e.g., 18 mg/ml) than females (e.g., 4.7 mg/ml) although this treatment usually results in a high mortality (50% by day 100) in female hamsters. This marked elevation of serum IgM is not attended by a similar increase in other serum Ig, although an occasional ascites observed in these animals contains high levels (8 to 10 mg/ml) of IgA and of 6 lymphomas detected after this treatment 4 were composed of IgG_2 B cells (one of these has been shown to secrete whole IgG_2 and free L chains). The enhanced IgM synthesis is especially apparent in spleen (by FA and C^{14} tissue culture analysis) and limited attempts in passive transfer of enhanced IgM synthesis into lethally irradiated recipients have been unsuccessful. The elevated IgM in some individual sera is of restricted electrophoretic heterogeneity which suggests a monoclonal origin, however definitive proof by production of idiotype specific antisera and isoelectric focusing of L chains are presently under investigation. This IgM has been shown to be of 19S size, although the specificity is unknown. The applicability of this IgM stimulation (?monoclonal induction) technique for other rodents is currently being evaluated. The mycobacterial factor necessary has been shown to be present in cell walls and is not in cell wall skeleton and is not MDP.

Structural analysis of female protein (FP). A sex limited serum protein of female Syrian hamsters (called female protein) has previously been defined in this laboratory. The generous quantities (2 mg/ml) of this hormonally controlled (testosterone suppression) serum protein suggests an important function although none has been defined at present. Analysis of its structure, however, indicates a strong similarity to C-reactive protein (CRP). That is, FP has been found to be composed of identical ∿25,000 molecular weight subunits noncovalently linked to form a protein of ∿150,000 molecular weight. In addition to CRP, this unusual structure has also been described for two other related proteins C1t (a complement component) and P-component of amyloid. In collaboration with Dr. John Sogn, we are now analyzing FP for amino acid sequence similarities to the above proteins. Because of the similarity of hormonal control of FP and of SLP (a marker of SS protein which is a complement component with an H-2 linked gene in mouse), we have investigated FP as a potential marker for the MHC locus of Syrian hamster. A search for antigenic or electrophoretic differences of FP among various outbred and inbred hamster strains, commercially available, showed no differences in these strains. However, among 9 inbred strains derived from a recent capture of wild hamsters in Syria, 3 strains were

found with an electrophoretically slower (but antigenically identical) FP. This electrophoretic difference presumably is due to protein differences, rather than sialic acid content as neuraminidase treatment causes a similar electrophoretic retardation in both slow and fast FP's. FP obtained from female (or castrated male) hybrids of fast/slow FP parents, show an intermediate mobility, suggesting fast/slow hybrid FP molecules. In collaboration with Dr. Wayne Streilein, we are comparing this serum FP marker with histocompatability data from backcross populations derived from fast and slow FP parents to determine if the FP gene is linked to the elusive MHC of hamsters.

Publications:

In press:

Coe, J. E.: Serum proteins: normal values derived from normal strains. FASEB Data Book - Inbred and Genetically Defined Strains of Laboratory Animals

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00074-07 LPVD

PERIOD COVERED: October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Host Defense Mechanisms in Chronic Viral and Neoplastic Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	J. K. Collins	Staff Fellow	LPVD NIAID
	W. J. Britt	Staff Fellow	LPVD NIAID

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Dr. Kenneth Watson, Dept. of Chemistry, University of Montana, Missoula, MT

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Laboratory of Persistent Viral Diseases, Hamilton, MT

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INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The main goal of this project is the understanding of host defense mechanisms involved in recovery from virus-induced leukemia in mice. We have used selected breeding of mouse strains to isolate and characterize individual genetically controlled defense mechanisms and to study their actions and interactions during recovery from virus infection and/or virus-induced leukemia. In addition, we have analyzed a group of leukemia cell variants with alterations in viral expression. These are being characterized biochemically and immunologically to determine the roles of expression of different viral proteins in immune recognition and the possible relevance of this variation in expression to persistence of virus infected cells in vivo.

PHS-6040
(Rev. 10-76)

Project Description:

We have previously identified 3 mouse genes (Rfv-1, Rfv-2, and Rfv-3) which are involved in host defenses against Friend virus (FV)-induced erythroleukemia. Rfv-1 is located in the H-2D region of the major histocompatibility complex (MHC), and has a major influence on the incidence of recovery from leukemia. The Rfv-2 gene is also associated with the MHC in the H-2K, H-2I or Tla regions, and has a weak effect on recovery from leukemia. The genetic linkage of the Rfv-3 gene is not known. It is not associated with H-2, however, it acts in a complementary fashion with the H-2-associated genes, Rfv-1 and Rfv-2, to influence recovery from leukemia. In the absence of the appropriate H-2 genotypes the Rfv-3 gene influences recovery from viremia, even in the presence of persistent leukemia. Our current work involves characterization of the mechanisms of action and roles of these genes in the host immune responses to virus and leukemia cells.

H-2-associated genes (Rfv-1 and Rfv-2). We have recently identified the organs in which H-2 effects on recovery from leukemia occur by use of irradiation chimeras. When spleen and bone marrow cells were transferred to lethally irradiated semi-allogeneic recipients ($H\text{-}2^{b/a} \rightarrow H\text{-}2^{b/b}$ and $H\text{-}2^{b/a} \rightarrow H\text{-}2^{a/a}$) the H-2 type of the donor spleen and bone marrow cells determined the incidence of recovery from FV leukemia. Thus H-2-associated effects appeared to be exerted by cells from these donor organs. Since both the leukemia cells and the cells of the immune defense system were derived from the donor stem cells in irradiation chimeras, we have attempted to distinguish which of these two general cell categories was the major site of H-2 influence on recovery. Two lines of preliminary evidence suggested that the main H-2 effects operated via the immune system rather than via the H-2 type of the leukemia cells. First, $H\text{-}2^{a/b}$ mice recovering from FV leukemia were able to reject transplanted FV leukemia cells of $H\text{-}2^{b/b}$, $H\text{-}2^{b/a}$ and $H\text{-}2^{a/a}$ genotypes with equal efficiency. Second, nonimmune $H\text{-}2^{b/b}$ spleen cells from mice made neonatally tolerant to $H\text{-}2^{a/b}$ cells were able to potentiate recovery of $H\text{-}2^{a/b}$ mice from FV leukemia. Thus, cells of the $H\text{-}2^{b/b}$ immune system were able to eliminate leukemia cells of the low recovery $H\text{-}2^{a/b}$ genotype. Our present preliminary conclusion is that the H-2 type of the immune system influences recovery from leukemia by immune response genes which control the rate of generation of FV-specific cytotoxic T lymphocytes which kill leukemia cells.

We have also approached the role of the immune response in recovery from leukemia by study of FV specific T cell proliferative responses in vitro. Preliminary data indicate that recovery from leukemia is associated with a strong T cell proliferative response, and current experiments are aimed at determining whether the lack of a similar response in persistently leukemic mice is due to suppression by virus or virus-specific suppressor cells. In additional studies (see below), the viral antigens recognized by both T and B cells are being studied.

Rfv-3 gene. 30 to 90 days after FV inoculation of Rfv-$3^{r/s}$ mice [(B10.A X A)F_1], we previously noted the association of elimination of viremia, loss of FV-induced cell surface antigens from splenic leukemia cells, and presence of cytotoxic anti-FV antibody in plasma. Furthermore, these parameters segregated together in a backcross between Rfv-$3^{r/s}$ and Rfv-$3^{s/s}$ mice. Thus, they appeared to be linked by a single genetically controlled mechanism. This mechanism probably involved control of production of cytotoxic anti-FV antibody, since passive transfer of these antibodies could reproduce the loss of FV cell surface antigens in vivo. Our recent experiments have shown that antibody-induced loss of viral cell surface antigens leads to a marked reduction in virus release by leukemia cells. This decrease in release is reflected as a 300-fold decrease in the percent of leukemia cells detectable as infectious centers. At this time cell-free virus in spleen homogenates is reduced as assayed both by infectivity and reverse transcriptase. Decreased virus release by persistent leukemia cells appears to result in elimination of viremia. Virus neutralizing antibodies do not seem to play a significant role. Our present evidence supports the idea that anti-FV antibodies reduce FV cell surface antigens by the process of antigenic modulation. We now hypothesize that antibody-induced rearrangement of viral cell surface molecules may interfere with important steric relationships among these molecules which in turn leads to reduced virus budding and release.

Current experiments are aimed at determining biochemically which viral cell surface molecules are decreased on these cells and also whether these cell surface events cause changes in intracellular viral protein synthesis and processing. Lastly, we are attempting to determine what role, if any, this phenomenon might have in interfering with or amplifying T cell-mediated mechanisms of specific immune recognition which could be important in elimination of leukemia cells.

Leukemia cell variants. We have obtained several lines of cloned FV leukemia cells which have variations in virus expression. These variant cells appear to have existed in the original leukemic mouse spleen from which they were derived since no further variations have been observed following cell cloning in vitro. These clones have been characterized for FV cell surface and intracellular antigens, reinfectibility, rescuability of spleen focus-forming virus, and recognition by antibodies and T lymphocytes from mice recovering from FV leukemia. Using these clones we have attempted to determine which FV-induced molecules are involved in different components of specific immune recognition in vivo. Analysis of the cloned variants obtained so far has also enabled studies on mechanisms involved in normal and aberrant viral protein synthesis and processing. These mechanisms may serve as interesting models because some of the "defects" leading to the nonproducer state may allow persistence of leukemia cells in vivo.

One clone has been found to be deficient in expression and/or processing of "gag" region viral proteins, whereas its expression of "env" region protein (gp70) is normal. Conversely, another clone makes no FV gp70, but appears to express normal amounts of the "gag" protein, p12. Using these two clones we have found that the FV-specific T cell proliferative response and cytotoxic T cell response are both mainly directed at the "env" protein, gp70. One "nonproducer" clone has been found to produce high levels of virus completely lacking reverse transcriptase yet containing normal levels of gp70. Also defective in this virus is an uncleaved "gag" polyprotein precursor. It is currently under investigation whether this is a single mutation or defect giving rise to pleiotropic effects on viral synthesis or whether this cell clone has multiple defects.

Other cells being analyzed by the above techniques include clones showing 1) deficient synthesis of viral core protein precursor synthesis, 2) expression of endogenous viral components in addition to Friend specific components, 3) expression of intracellular viral antigens which are not processed to cell surface antigens, and 4) possible alteration in expression of cellular H-2 antigen determinants.

Publications:

Doig, D. and Chesebro, B.: Antibody-induced loss of Friend virus leukemia cell surface antigens occurs during progression of erythroleukemia in F_1 mice. J. Exp. Med. 148: 1109-1121, 1978.

Chesebro, B. and Wehrly, K.: Identification of a non-H-2 gene (Rfv-3) influencing recovery from viremia and leukemia induced by Friend virus complex. Proc. Natl. Acad. Sci. USA 76: 425-429, 1979.

In press:

Chesebro, B., Wehrly, K., Doig, D. and Nishio, J.: Antibody-induced modulation of Friend virus cell surface antigens decreases virus production by persistent erythroleukemia cells: Influence of the Rfv-3 gene. Proc. Natl. Acad. Sci. USA.

Doig, D. and Chesebro, B.: Anti-Friend virus antibody is associated with recovery from viremia and loss of viral leukemia cell surface antigens in leukemic mice. Identification of Rfv-3 as a gene locus influencing antibody production. J. Exp. Med.

Chesebro, B.: The influence of the major histocompatibility complex (H-2) on oncornavirus-induced neoplasia in mice. In Kaiser, H. E. (Ed.): Comparative Pathology of Abnormal Growth.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00085-02 LPVD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Biology of Aleutian Disease Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	J. E. Coe	Medical Officer	LPVD NIAID
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2.2	1.2	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project is to study Aleutian disease (AD), a severe, fatal immune complex disease of mink associated with a persistent viral infection. Techniques of both virology and immunology are focused on this problem. The current objectives are to characterize the Aleutian disease virus (ADV) and to determine what role viral components have in the genesis of the immune complexes.

PHS-6040
(Rev. 10-76)

Project Description:

Previous work at RML by this PI and others showed that the Utah I strain of Aleutian disease virus (ADV) could be successfully purified from in vivo grown materials by equilibrium ultracentrifugation in cesium chloride density gradients, if virus was first released from naturally occurring immune complexes. These studies suggested, on the basis of size, morphology, and density that ADV was probably a member of the parvovirus group, small icosahedral viruses that encapsidate single stranded DNA genomes. Recently, Utah I ADV has been adapted to growth in cell cultures of Crandall feline kidney cells (CRFK) at 31.8°C and assayed, using immunofluorescence.

We have now characterized one cell culture adapted strain of ADV originally derived from Utah I ADV by Dr. John Gorham at Washington State University. This strain of ADV (ADV-Gorham), which grows in Crandall feline kidney cells (CRFK) to high titer, has been examined at several in vitro passage levels. At the 5th CRFK passage, the virus had an in vitro titer of $10^{8.04}$ fluorescence-forming units per ml (FFU/ml), an in vivo titer (based on induction of antibody response in mink) of $10^{6.30}$ infectious dose 50% per ml (ID_{50}/ml), and caused typical AD in some mink. However, at the 10th passage in CRFK, although the in vitro titer was $10^{7.40}$ FFU/ml, the in vivo titer was $10^{1.5}$ ID_{50}/ml and the virus was nonpathogenic. Viral growth studies in vitro showed that virus production was equivalent at 31.8° or 37°C for 2 days after which time virus yield at 37°C decreased. This suggested that at 31.8°C, the virus only proceeds through a single cycle of infection. Virus from infected cell pellets could be purified on CsCl gradients revealing a major peak of infectivity at a density of 1.42 to 1.44 gm/ml and a minor peak at 1.34 to 1.35 gm/ml. Viral antigen was detected in both peaks by counterimmunoelectrophoresis using 1) AD mink serum, 2) rabbit sera prepared against CsCl purified in vivo virulent ADV or 3) rabbit sera prepared against CsCl purified cell culture adapted ADV, thus suggesting that the cell culture virus is in fact related antigenically to ADV.

Experiments have also been done with isotopic precursors, and ^{3}H-thymidine could be incorporated into both 1.42 to 1.44 gm/ml and 1.34 to 1.35 gm/ml peaks of infectivity. When DNA from the major peak was analyzed in alkaline sucrose gradients, a single homogeneous species of approximately 15S was detected. The above findings strongly suggest that this virus is a parvovirus.

We have also done preliminary characterization of another cell culture adapted strain of Utah I ADV derived by Dr. David Porter of UCLA School of Medicine. This virus differs markedly from the ADV-Gorham isolate in that it is extremely virulent for mink. Comparative in vivo and in vitro titrations showed an ID_{50} of 10^{6} with all mink developing clinical Aleutian disease, but an in vitro titer of only 2×10^{4} FFU/ml. This low in vitro titer has seriously hampered characterization so far.

In addition, we have also been able to adapt the in vivo Utah I ADV to CRFK cell culture passage in this laboratory and are undertaking studies similar to those described above with this strain as well.

Future plans for this project may be broken down as follows: 1) We plan collaborative studies with Dr. David Ward of Yale University School of Medicine to define the exact structure of the ADV-Gorham viral DNA and its homology relationship to DNA's of other nondefective parvoviruses. These experiments will involve nuclease digestions, restriction endonuclease analysis, heteroduplex mapping, and will provide definitive assignment of ADV to the parvovirus group. Furthermore, the reagents developed in these studies will allow us to compare the DNA of the cell culture adapted ADV-Gorham strain, to DNA from in vivo virulent ADV as well as that from other cell culture derived strains of ADV. 2) We plan to compare the polypeptide composition of both cell culture adapted and in vivo strains of ADV by both extrinsic (^{125}I) and intrinsic (^{35}S) labeling and subsequent analysis on SDS polyacrylamide gels. Thus, we will be able to define the viral protein structure and examine the antigenicity of each polypeptide constituent. 3) A solid phase radioimmunoassay is currently under development in an attempt to enhance the sensitivity of detecting both viral antigens and antiviral antibodies over that afforded by CIE. 4) By comparing the macromolecular composition of the various strains of ADV, we may be able to delineate differences between virulent and nonvirulent strains of this virus.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00086-02 LPVD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Mechanisms of Immune Recognition of Viral Antigens in Persistent Viral Diseases

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2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major goal of this project is the definition of mechanisms of immune recognition of viral antigens on the cell surface of virus infected cells. We are continuing to study the murine leukemia line FBL-3 which undergoes a major phenotypic alteration in vivo converting from benign to malignant behavior. Cells grown in vitro or for 7 days in the ascites form produce transient tumors when injected subcutaneously. The cells passed in ascites for 14 days produce lethal subcutaneous tumors which metastasize. We have found by fluctuation analysis that malignant tumor cell variants exist in the parent line and that as yet undefined selective pressure in the host allows the preferential expansion of the pre-existing variants. Detailed studies of the surface antigens expressed by cloned tumor cell variants have revealed important aberrations in the major histocompatibility antigens expressed by these cells which may adversely affect their immune recognition by the syngeneic host.

PHS-6040
(Rev. 10-76)

Project Description:

FBL-3, a C57BL/6 ($H\text{-}2^{b/b}$) murine leukemia was originally induced by Friend virus and expresses MuLV (the helper virus) but not the defective SFFV. The tumor cells grown in vitro or for 7 days in the ascites form produce transient tumors when injected subcutaneously (s.c.). These tumors are uniformly rejected by the syngeneic host. However, after 14 days in the ascites form the tumor cells were found to have converted to a highly malignant form which grew progressively after s.c. injection, disseminating to local and distant lymph nodes (occasionally to the liver) and was lethal within 40 to 50 days.

In the past year we have examined two questions: 1) Is the phenotypic alteration the result of the expansion of a population of malignant tumor cell variant pre-existing in the parent tissue culture line or did the variants arise de novo? 2) What is the nature of this phenotypic change specifically with respect to H-2 and viral antigen expressed on the cell membrane?

Evidence for pre-existing malignant variants. To approach this problem we utilized the technique of fluctuation analysis which has been applied extensively in the study of bacterial mutants. The in vitro grown parent line of FBL-3 (which is uniformly rejected by syngeneic recipients after s.c. inoculation) was cloned and the individual clones were tested for tumorigenic potential. Of 9 clones, 5 showed malignant behavior and 4 were benign. This strongly suggests that malignant variants do pre-exist in the parent population and that as yet unknown in vivo selective pressures allow for their expansion between the 7th and 14th day in the peritoneal cavity. The relatively high frequency of malignant/benign clones was unexpected but may be explained by a higher cloning efficiency of the malignant variants. We are currently, with the help of a summer student, repeating the fluctuation analysis with larger numbers of animals in order to determine if there are any differences in tissue tropism of those malignant variants which metastasize. With the phenotype of each clone defined we plan to study their cell surface expression of viral and MHC antigens which might explain differences in their immune recognition by the host.

Mechanisms of immune recognition by the host of tumor cell variants. To this end we have been studying the expression of cell surface antigens by an unusual cloned variant (FY7) of FBL-3 ($H\text{-}2^{b/b}$). This variant was derived from cells that were grown for 7 days in the ascites form in $(B10.A \times A.BY)F_1$ mice ($H\text{-}2^{a/b}$). This clone is highly immunogenic and provokes a potent anti-FBL-3 response when injected s.c. Immune peritoneal exudate cells (PEC) derived by secondary intraperitoneal challenge of FY7-immune mice are cytotoxic for FY7 ascites cells. The effector cell in the PEC population has been identified as a cytotoxic T lymphocyte (CTL). However, these CTL fail to lyse FY7 which has been maintained in vitro. Furthermore, in vitro FY7 fails to competitively inhibit the lysis by CTL of

ascites FY7 indicating that in vitro FY7 does not express the relevant antigens recognized by CTL. We have concentrated our efforts on the in vitro line since there is no problem with host cell contamination, and have examined the cell membrane expression of FBL-3 neoantigens and MHC determinants. Anti-H-2^b CTL generated by primary in vitro culture of (B10.A X A)F_1 (H-$2^{a/a}$) or (B10.D2 X BALB/c)F_1 (H-$2^{d/d}$) responders with mitomycin-treated (subscript mc) B10 spleen cell stimulators fail to lyse FY7 even at an attacker/target ratio of 100/1. Yet these CTL do lyse other H-$2^{b/b}$ tumor cell lines and B10 splenic con A blasts. In addition FY7 does not competitively inhibit the lysis of B10 con A blasts by anti-H-2^b CTL. Thus, FY7 appears not to express the relevant H-2^b determinants recognized by anti-H-2^b CTL. We found using serologic techniques that FY7 expresses quantitatively similar amounts of H-$2K^b$ serologic specificities but completely lacks H-$2D^b$ determinants detectable by antibody. In order to rule out the possibility that anti-H-2^b CTL recognizes only H-$2D^b$, we generated CTL using B10.A(4R) (K^kD^b) responders stimulated by $B10_{mc}$ (K^bD^b). Even with CTL with specificity to H-$2K^b$ FY7 fails to competitively inhibit. This suggests that the H-$2K^b$ determinants detected by antibody are distinct from those detected by CTL and that FY7 expresses the former but not the latter. Although the association between lack of H-$2D^b$ serologic and H-$2K^b$ CTL specificities on FY7 may be coincidental, it raises an interesting question. Recently, the biosynthesis of an I region antigen (IE^k) has been found to be dependent on the expression of two separate I region genes IA^k and IE^k. As yet we have no evidence for multi-gene control of H-$2K^b$. However, FY7 is unique in that it is, to our knowledge, the only homozygous H-2^b cell line that fails to express an H-2D product and therefore provides a useful tool to study this possibility.

The lack of recognition on FY7 of FBL-3 neoantigens by anti-FBL-3 CTL despite their detection by anti-FBL-3 antibody correlates with the aberrations in expression of H-2 antigens. The phenomenon of H-2 restriction in the recognition of viral, chemical and tumor associated cell-membrane antigens has been extensively documented. A small number of tumor cell variants have been described which lack the expression of any H-2 antigens detected by serology or CTL. CTL fail to recognize antigens chemically coupled to these cells but do recognize these determinants on the parent populations which do express H-2. Since FY7 is selectively missing an H-$2D^b$ gene product we are examining other variants of FBL-3 in order to establish whether there is a correlation between the lack of H-$2D^b$ and absence of CTL recognition of neoantigens on these cells.

An approach to the difference between FY7 grown in ascites vs. in vitro is more difficult. We are interested in determining if H-$2D^b$ is expressed on the ascites cells. However, because of significant contamination of the ascites tumor cells with host cells (up to 40%) we are attempting to develop cell separation procedures which will allow this comparison. It is clear, however, using crude separation on nylon wool and limiting dilution techniques, that there is a distinct difference between the expression of tumor specific CTL recognition determinants on ascites vs. in vitro grown cells.

Studies on the H-2K^b product of FY7. Much of the biochemical analysis of H-2 gene products has been carried out on H-2 mutant mouse strains and on well defined tumor cells such as the C57BL/6 (H-2$^{b/b}$) leukemia line EL-4. Ultimately these studies are directed toward defining the structural correlates of the various biologic functions of H-2 gene products. Since FY7 presents a unique aberration in its expression of H-2K^b specificities we are comparing the K^b product of FY7 with that of the well characterized line EL-4. Using the technique of immunoprecipitation and resolution by SDS polyacrylamide electrophoresis (PAGE) the K^b product appears to have the same molecular weight ($\sim$44K) as that of EL-4 and BL/10 spleen cells. Further resolution by two dimensional (2D) PAGE will allow comparison of the various polypeptides within this 44K band. We are currently producing hybridomas with anti-H-2K^b and H-2D^b specificities in order to facilitate selective precipitation of these products. This is particularly important for 2D gels since a wide array of polypeptides are resolved by this technique and much confusion can be eliminated by using monoclonal antibodies of defined specificity. This work is being done in collaboration with Drs. John Colligan and Thomas Kindt (Laboratory of Immunogenetics) who are looking for evidence of primary sequence differences between the K^b product of FY7 and EL-4.

Biochemical studies of H-2 variants will allow more precise analysis of the structural nature of CTL specificities and may also provide information on the H-2 determinants recognized by viral specific T lymphocytes. We plan to select for such variants in vitro by repeated exposure of tumor cells to anti-H-2 CTL. This approach should allow the screening of large numbers of tumor cells for infrequent aberrations in their expression of H-2 determinants recognized by CTL.

Publications:

Portis, J. L.: Changes in the transplantability of a virus-induced murine leukemia tumor. J. Natl. Cancer Inst. 62: 611-617, 1979.

Portis, J. L., Wikel, S. K. and McAtee, F. J.: A simplified rapid method for purification of glomeruli. J. Clin. Pathol. 32: 406-409, 1979.

In press:

Portis, J. L. and Coe, J. E.: Deposition of IgA in renal glomeruli of mink affected with Aleutian disease. Am. J. Pathol.

EPIDEMIOLOGY BRANCH
Rocky Mountain Laboratories
Hamilton, Montana
1979 Annual Report
Table of Contents

Annual Report
Epidemiology Branch
Rocky Mountain Laboratories
Hamilton, Montana
National Institute of Allergy and Infectious Diseases
October 1, 1978 to September 30, 1979

RESEARCH HIGHLIGHTS

The following sections summarize highlights of our research program and discuss their significance in relationship to the epidemiology and ecology of the diseases under investigation. Some of these findings and others were reported in 20 publications. An additional 30 papers have been accepted by scientific journals.

RICKETTSIOSES

Collaboration with various universities and health agencies in the United States and abroad has continued to provide clinical and field materials vital to development of information on distribution and nature of tick-borne rickettsiae and rickettsial infections. Consequently, we have gained insight as to the variety of spotted fever-group (SFG) rickettsiae encountered in ticks parasitic for man in the United States. We can now suggest preliminary hypotheses regarding distribution of these agents, their ecologic relationships to Rickettsia rickettsii, and their role as causes of human infection.

Earlier tick surveys have provided information about the prevalence and serotypes of SFG rickettsiae in Connecticut, Massachusetts, New York, South Carolina, Tennessee, Alabama, Mississippi, Ohio, Oregon, and Montana. Current studies will give additional information about the variety and nature of rickettsiae encountered in Dermacentor occidentalis and Ixodes pacificus from California and D. variabilis from North Carolina. The number of SFG serotypes in the U.S. is probably finite (10 have been identified to date). Four are broadly disseminated in both eastern and western states and occur in more than one species of tick-vector of Rocky Mountain spotted fever (RMSF). These include R. rickettsii, R. montana, R. rhipicephali and the unclassified 369-C agent. Only R. rickettsii has thus far been etiologically associated with human illness, and indications are that the other three are avirulent for man (as well as for experimental animals) although tantalizing evidence based on serologic responses in residents from Long Island and California suggests that inapparent or missed infection may sometimes occur.

There is also fragmentary epidemiologic evidence to suggest that nonpathogenic members of the SFG may have bearing on the focalization of R. rickettsii. Usually, R. rickettsii is seldom found in areas where prevalence rates of other members of the SFG are high. We are considering

the possibility that related nonvirulent rickettsiae compete in mammalian amplifying hosts or otherwise interfere with the maintenance cycle of R. rickettsii, perhaps by blocking transovarial transmission in the arthropod host. These possibilities can be investigated in the laboratory and will occupy much of our attention during the next several years.

An increasingly crucial question has to do with the stability of SFG rickettsiae. It has been assumed on basis of unchanging characteristics of rickettsiae under laboratory conditions that antigenic differences encountered in nature are slowly evolving manifestations and are not merely expressions of short term phase variation or degradation induced by stresses of uncertain existence in the arthropod host under varying environmental influences. However, it is increasingly evident that the tick host is the keystone to our understanding of the ecology of R. rickettsii. We know very little about host-parasite relationships at the cellular and subcellular level. We will need additional expertise to define host-parasite interactions, morphologically by electron microscopy, and functionally by tick physiology, biochemistry, and molecular biology. These needs apply to tickborne viruses as well.

Specifically, a hemolymph-test survey in four areas of North Carolina revealed SFG rickettsiae in 0.6 to 12.4% of 2,000 D. variabilis examined. Isolation and identification of these organisms are pending. Rickettsiae were detected in as many as 35% of Amblyomma americanum collected in certain southeastern areas of the United States. One characterized strain was antigenically distinct from any hitherto described SFG rickettsia. Differences were also detected by electron microscopy, pathogenicity for experimental animals, and protein composition. The collaborative study with the Harvard School of Public Health of D. variabilis on Cape Cod was concluded. Forty-three isolates of SFG rickettsiae were recovered from 7,000 ticks. Thirty-nine were R. montana, one was R. rickettsii and three were unclassified. Etiologic association of R. montana with RMSF could not be demonstrated. A rickettsia comprising a new SFG serotype was recovered from I. ricinus in Switzerland. The "Swiss agent" is pathogenic for meadow voles, chick embryos, and several lines of tissue culture cells, but not for guinea pigs. It possesses antigen(s) common to the SFG but is sharply distinct from R. rickettsii, R. sibirica, R. slovaca, and R. conorii. D. andersoni collected on the east side of the Bitterroot Valley, Montana, an area free of R. rickettsii, frequently contain rickettsiae in ovarial tissues which are nonpathogenic for various experimental animals and indigenous rodents. It is hypothesized that this organism is maintained solely by transovarial passage and may render its arthropod host refractory to established infection by R. rickettsii. (Burgdorfer)

Eight isolates of SFG rickettsiae obtained by cell culture during the 1977 Bitterroot Valley survey of D. andersoni ticks, two representing each of four serologic types identified by microimmunofluorescence (micro-IF) including R. rickettsii, R. montana, R. rhipicephali, and the

unclassified 369-C agent were examined for other distinguishing biologic characteristics including pathogenicity for chick embryos, guinea pigs, and cell culture; stability during serial passage in Vero cells; antibiotic sensitivity; cross-immunogenicity in guinea pigs; and growth dynamics in cell culture. Strains within serotypes were similar, but the serotypes could be readily distinguished one from another on basis of several biologic markers in addition to serologic reactivity. These findings give us added confidence in the micro-IF test as a practicable procedure for differentiating rickettsiae. In collaboration with the California State Department of Health, we are determining the kinds of rickettsiae prevalent in D. occidentalis and I. pacificus in several coastal areas where sporadic cases of RMSF occur in absence of the usual tick vectors. Rickettsiae representing four serotypes have been isolated; one similar to R. rickettsii, one related to the 369-C agent, one indistinguishable from an unclassified SFG rickettsia found in I. pacificus ticks in Oregon, and numerous isolates similar to R. rhipicephali. (Philip)

In western Montana, two of three persons bitten by R. rickettsii-infected ticks acquired RMSF. On the other hand, 13 persons bitten by ticks infected with R. rhipicephali, R. montana, or the 369-C agent were not ill and did not develop antibodies against these organisms. However, in a collaborative study in New York state, 14% of 103 residents of Shelter Island at the eastern tip of Long Island developed low but significant levels of micro-IF antibodies to R. rickettsii and/or the 369-C agent between May and October 1978. Since illness could not be correlated with antibody responses, the source of the reactivity is enigmatic. Similar low levels of antibody are occasionally found among coastal residents of California with tick-associated illnesses suggesting that other SFG agents may occasionally give rise to inapparent infection or abortive disease. We are continuing our investigations on the serodiagnosis of rickettsial disease. A latex agglutination test (in collaboration with the New York State Health Department), an enzyme-linked immunosorbent assay (ELISA), and a fluoroimmunometric assay (FIAX) were evaluated for the diagnosis of RMSF. The last two tests are quantitative and 4 to 8 times more sensitive than our standard micro-IF test, but otherwise offer few advantages over the latter. (Philip, Casper)

LEGIONNAIRES' DISEASE

Studies on Legionnaires' disease (LD) are continuing, on reduced scale after the retirement of Dr. Ormsbee. We hope to increase activity in this area in the near future. The parameters of infectivity and pathogenicity of four strains (LAC, LAP, LAW, LAB) of Legionella pneumophila representing three serologic types initially cultivated in chick embryo yolk sac were determined after 9 and 12 serial passages on Mueller-Hinton (MH) medium. The plaquing ability on chick embryo cells and pathogenicity for guinea pigs of the LAC, LAP, and LAB strains was greatly diminished after in vitro passage. The LAW strain, originally isolated on MH medium,

Legionnaires' Disease (cont'd)

showed good plaquing ability that did not change during passage. This strain was only weakly pathogenic for guinea pigs both before and after passage. We are now looking for biochemical and serologic markers to explain the changes noted. Further passages in chick-embryo yolk sac are also being conducted to determine whether variations are stable characteristics. Work with LD during the coming year will include development of better laboratory procedures for early diagnosis and definition of growth dynamics of L. pneumophila in chick-embryo cell culture. (Ormsbee, Peacock)

SYSTEMATICS OF TICKS

Systematic treatment of ticks of medical importance, including taxonomy, biology, ecology and colonization continues to be the major goal of this project. Our studies on the taxonomy of the genus Argas based on the morphology of Haller's organ have been expanded to include more species in each subgenus. We hope that morphology of this organ can be correlated with host-specificity and other behavioral characteristics of these ticks. Significant progress was made in our continuing study of the Ornithodoros capensis complex. Using the scanning electron microscope (SEM), we have redescribed O. amblus, an ectoparasite of marine birds in Peru, and are preparing photos of O. muesebecki for possible redescription of this species. Both ticks frequently bite man and carry viruses that may cause disease in humans as well as birds. We are also completing descriptions of O. darwini and O. galapagensis, ectoparasites which are probable vectors of intraerythrocytic hemogregarines of marine iguanas and lava lizards on the Galapagos Islands. Practical guides, amply illustrated with SEM photos that will allow easier identification, have been prepared for the genus Ixodes and are being prepared for Dermacentor as well. The magnification of the photos is well within the range of standard stereoscopic microscopes and should enable health authorities to readily identify vectors of RMSF. We initiated a study to clarify the status of an Ixodes species of the angustus complex that occurs in Black Hills, South Dakota, where years ago, Dr. Carl Eklund isolated Powassan virus from Ixodes. We are also continuing our investigations of medically and agriculturally important rhipicephalid species from Africa and hope soon to initiate a study of the R. sanguineus complex, some members of which are vectors of human rickettsioses. Final editing of the master list of Smithsonian computer data on the RML-NAMRU-3 tick collection has been completed. This fall, a mini file will be prepared for economy of data-search, and we can begin to use the information. In the meantime, new records are rapidly accumulating and we have as yet no means for updating the computer data bank on a continuing basis. (Clifford, Keirans)

Results of research on the G.H.F. Nuttall tick collection, the world's third largest, deposited in the British Museum, are being compiled for publication in book form. This collection contains over 100 type-

species that are known vectors of human and animal disease agents. Many are in need of taxonomic clarification. A second important tick collection, that of N. C. Rothschild, was also redetermined and updated. (Keirans)

In taxonomic studies of mites, manuscripts describing clinical mange in the brown bear and laboratory primates, as well as the macronyssid fauna of Surinam, have been submitted for publication. Parasitic Acari of Saudi Arabia are being studied preliminary to preparation of a fauna list and taxonomic key for public health workers. (Yunker)

ARTHROPOD-BORNE VIRUSES

In our continuing studies of distribution, ecology, identification, characterization and classification of tick-borne viruses and their potential relationship to human disease, 102 isolates were obtained from more than 1,100 ticks collected in seven states. Agents identified thus far included 30 isolates of Soldado virus from Ornithodoros capensis ticks in Texas, 25 strains of Hughes virus from O. denmarki in Florida, 15 strains of Sapphire II virus from Argas cooleyi in South Dakota, and 16 isolates of Sixgun City virus from the Argas collection. Both species of Ornithodoros are parasites of migratory seabirds. Soldado and Hughes virus are distantly related members of an unclassified taxon referred to as Hughes virus serogroup. Some Hughes group agents have been implicated in seabird die-offs and febrile diseases of man. A. cooleyi is a parasite of migratory passarine birds, and Sapphire II virus is also a member of the Hughes serogroup. Sixgun City is a reovirus. Geographical records of tick-borne viruses in various vectors and in flyways of migratory birds enable us to predict distribution and occurrence of these pathogens. High prevalence of several of these viruses in ticks (e.g., Soldado and Hughes viruses) suggests that these arthropods serve both as host and vector. Of particular interest was the recent isolation of an unidentified virus from D. occidentalis collected in two Oregon counties. D. occidentalis is abundantly distributed in coastal areas from Washington to California and frequently bites man. Fourteen isolates were obtained in a poikilothermic cell line derived from Xenopus laevis, South African clawed frog. None was obtained in Vero cells. The virus is pathogenic for meadow voles and will propagate in L cells, pig kidney cells and tick tissue cultures, but it is non-pathogenic for suckling mice and hamsters. Physicochemical properties are being characterized. Xenopus cells were also superior to other lines for recovery of Soldado virus. It may prove particularly useful for isolation of temperature-sensitive variants. (Yunker, Clifford, Thomas)

Our studies of vector-borne virus interaction with arthropods, particularly ticks at a cellular level, continue. Last year, we began investigation of transovarial transmission of Flaviviruses by ticks. Langat virus inoculated parenterally into Dermacentor females was transmitted by bite by all stages of the F_1 generation. However, virus could be demonstrated only in a few F_2 eggs and larvae and could not be detected

at all in subsequent stages. It may be that proviral DNA was not involved in this particular system in contrast to findings by Soviet investigators of DNA transcripts of Bhanja virus RNA in eggs of Hyalomma ticks. Additional experiments with Quaranfil virus in Argas arboreus and Colorado tick fever (CTF) virus in Dermacentor are underway. Carrier cultures of CTF virus were established in Xenopus cells grown at 27°C. Even after 50 passages apparently normal cultures yielded low titers of CTF virus which, unlike that grown in mosquito cells, was unchanged in pathogenicity for suckling mice. Thus, temperature of incubation alone does not seem to determine CTF virus attenuation in insect cells. Mosquito cell cultures were evaluated for detection of dengue viruses. Four serotypes of dengue virus were inoculated into mosquitos by Dr. Rosen in Hawaii. Virus was detected at RML from all but 3 of 24 infected mosquitos after they were inoculated into Aedes albopictus cells. Two strains missed were dengue-3 virus. Thus, it appears that mosquito cells may be useful for rapid detection of 3 of 4 dengue serotypes. This year, we have established a serially propagating cell line from D. variabilis embryos. These cells are now in their 21st passage, are diploid, and form monolayers within one week. The North American tick-borne Flavivirus, Powassan, was shown to replicate in these arthropod cells. Transovarial transmission of Flaviviruses by ticks, particularly St. Louis encephalitis virus, will receive particular emphasis next year, based on the recent discovery by French investigators of natural infections of yellow fever in, and transovarial transmission of virus by, wild-caught African ticks. (Yunker)

PATHOGENESIS OF SLOW VIRAL DISEASES

This project, which considers the unusual host-virus relationships that give rise to slowly evolving virus diseases after a long incubation period, is being terminated. Emphasis has been directed particularly to scrapie-like diseases of sheep, goats, mink and man. No new experiments were initiated during the year. However, a most noteworthy finding occurred in experiments still in progress. Four years ago, a pilot study was begun during which specimens of brain from 11 patients dying of Creutzfeldt-Jakob disease (CJD) were inoculated into brains of African pygmy goats. One of three goats that received one brain specimen and one of four that received another manifested a scrapie-like disease 43 months after inoculation. One goat was sacrificed 8 weeks after clinical signs appeared. Histologic examination of the brain and spinal cord showed changes compatible with those of caprine scrapie. Astrocytosis was prominent in diencephalon, mid brain, and cerebellar cortex, but spongiform changes were minimal. The other goat with scrapie-like signs is still being observed, and three penmates may now be in prodromal stage of disease. Goats inoculated with the other nine specimens are still normal, but most have not been observed long enough to warrant conclusions as to whether they too may become affected.

Occurrence of scrapie-like disease in these goats is a very significant finding in that it indicates a possible relationship of CJD virus to scrapie virus. Although CJD virus and scrapie virus induce similar neurologic diseases in squirrel and rhesus monkeys, the observation here that CJD virus induced disease and pathologic changes in goats that are indistinguishable from those resulting from natural infection with scrapie virus may be more significant than occurrence of similar disease in an aberrant host. Guinea pigs and mink inoculated intracerebrally with brain specimens from the same patients with CJ disease have remained free of recognizable neurologic disease after 2 and 4 years, respectively. A presumed neurologic disease in similarly inoculated mice has recurred in some of those in second passage, but the results are still inconclusive.

Except for continuing observation of animals inoculated with CJD material, no new experiments are currently contemplated. However, it is most important that the findings of scrapie-like disease in CJD virus-inoculated goats on primary passage of brain material from patients be confirmed by secondary passage in these animals. (Hadlow)

Annual Report
Epidemiology Branch
Rocky Mountain Laboratories
Hamilton, Montana
National Institute of Allergy and Infectious Diseases
October 1, 1978 to September 30, 1979

ADMINISTRATIVE REPORT

In March 1979, the Epidemiology Branch (EB) was officially established as one of three laboratories which comprise what was formerly the Rocky Mountain Laboratory. Currently, the EB comprises three sections, viz.: Arthropod-Borne Diseases, Epidemiology, and Histopathology. The EB is manned by 8 Ph.D.-level scientists, including 4 in the Arthropod-Borne Diseases Section, 3 in the Epidemiology Section, and one in the Histopathology Section. In addition, there are 12 supporting staff and one secretary assigned to the Office of the Chief. A permanent Chief of EB has not yet been appointed. The total complement is 22. All, at present, are tenured personnel. Prior to reorganization, two scientists retired (Dr. Richard Ormsbee in January 1979 and Dr. J. Frederick Bell in February 1979). Dr. Ormsbee's project on Legionnaires' disease is being continued and hopefully will be expanded in the near future. Dr. Bell's projects on rabies and tularemia have been terminated.

A visiting scientist, Dr. Klaes Kindmark, Uppsala, Sweden, arrived in February for a 6-month period in Dr. Burgdorfer's laboratory. He is developing more satisfactory techniques for early diagnosis of rickettsial diseases. Dr. William Todd, Colorado State University, will spend 2 years in Dr. Burgdorfer's laboratory doing tick-transmission studies of SFG rickettsiae under an IPA contract.

The EB has assumed the role of WHO Collaborating Center for Rickettsial Reference and Research which was formerly the responsibility of RML. In this regard, we provide reference rickettsial antigens and antisera and consultative expertise to qualified foreign as well as domestic investigators.

An International Symposium on Spongiform Encephalopathies was held at RML in honor of Dr. Carl Eklund, October 1978. Approximately 57 visitors (U.S. and foreign) attended. In June 1979, a Workshop on St. Louis Encephalitis was held at RML under sponsorship of the Viral Diseases Panel, U.S.-Japan Cooperative Medical Sciences Program, NIAID. Approximately 54 participants and 4 staff members of NIH attended this meeting.

Annual Report
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HONORS AND AWARDS

The following activities reflect the recognition afforded staff of EB by their peers in the scientific community.

Editorial Boards of Journals:

Dr. W. Burgdorfer - Acta Tropica
Dr. C. M. Clifford - Journal of Medical Entomology
Dr. C. E. Yunker - Journal of Parasitology

Drs. Burgdorfer, Yunker, Clifford, Hadlow, and Philip reviewed manuscripts submitted for publication in J. Med. Entomol., Am. J. Trop. Med. Hyg., J. Parasitol., Ann. Entomol. Soc. Am., Vet. Pathol., New Eng. J. Med.

Professional Posts:

Dr. R. N. Philip - Secretary, American Committee on Rickettsiae and Rickettsial Diseases

Dr. W. Burgdorfer - Consultant, University of Neuchatel, Switzerland

Dr. W. J. Hadlow - Member of the Council, American College of Veterinary Pathologists

Dr. C. E. Yunker - U.S. representative to the Committee on Polar Viruses

Invited Lectures and Participation in Meetings and Symposia:

Dr. W. Burgdorfer was invited by Rockefeller Foundation to participate and present a paper at the Conference on Comparative Aspects of Animal and Plant Pathogen Vectors, Bellagio, Italy; he was also invited by ASM Committee on Pathogenic Microorganisms to present a lecture on "Borreliae and Borrelioses", Los Angeles.

Dr. R. N. Philip presented a paper and chaired a session on rickettsiae at VII Intl. Congress of Infections and Parasitic Diseases, Varna, Bulgaria.

Dr. W. J. Hadlow headed the Organizing Committee of Symposium on Spongiform Encephalopathies, Hamilton, Montana, in honor of Dr. Carl Eklund.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00061-17 EB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Natural History of Tick-Borne Rickettsial Agents and Their Public Health Significance

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. Burgdorfer	Res. Entomologist (Med.)	EB NIAID
OTHER:	R. N. Philip	Medical Director	EB NIAID
	L. A. Thomas	Research Microbiologist	EB NIAID
	E. A. Casper	Nurse Director	EB NIAID
	R. L. Anacker	Research Microbiologist	LMSF NIAID

COOPERATING UNITS (if any) Dr. J. L. Benach, State of New York Dept. of Health; Dr. J. N. MacCormack, CDC Branch, Dept. Human Resources, North Carolina; Dr. W. Feng, Harvard School of Pub. Hlth.; Drs. J. Anderson and L. Magnarelli, Conn. Agric. Res. Exp. Station, Dr. C.-O. Kindmark, Uppsala, Sweden.

LAB/BRANCH
Epidemiology Branch (RML), Hamilton, MT 59840

SECTION
Arthropod-Borne Diseases Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.6	1.0	2.6

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is concerned with studies of Rocky Mountain spotted fever and other tick-borne rickettsial diseases in the United States and in other countries regarding ecology, identification, and characterization of the rickettsial agent(s) with their associated vectors. With collaboration of outside agencies (state health departments, public health laboratories, hospitals, physicians, etc.) source material for comparative studies on the various rickettsial types and information about the distribution of vector species associated with spotted fever in the United States is obtained. This project also considers the cellular and subcellular aspects of interaction between tick-borne agents, particularly rickettsiae, and their vectors.

PHS-6040
(Rev. 10-76)

Project Description:

Rocky Mountain spotted fever continues to be a significant health problem throughout most of the United States. As a result, many outside agencies called on this laboratory for guidance and assistance in their efforts to determine the epidemiologic, ecologic, and health impacts on the public. Because of changes in RML's rickettsial research program, some of our previous collaborative investigations with outside agencies had to be terminated or were limited to providing research guidance and/or diagnostic reagents necessary for epidemiological and ecological investigations. With the exception of a tick/rickettsial survey in North Carolina, most of our current projects are related to the evaluation and characterization of rickettsial material collected from various parts of the U.S. during recent years.

Research accomplishments during the past year include the following findings:

North Carolina continues to have the highest incidence of spotted fever cases in the U.S. In 1978, it reported 204 cases or approximately 20% of the nation-wide reported total. In cooperation with the Duke University Medical Center at Durham, N.C., and the Communicable Disease Control Branch, Department of Human Resources at Raleigh, N.C., a study was initiated to determine in selected areas of that state the prevalence of spotted fever group (SFG) rickettsiae, particularly of Rickettsia rickettsii in the American dog tick, Dermacentor variabilis. So far, 2,123 adult ticks from 4 different areas were examined. The percentage of ticks infected with typical pleomorphic SFG rickettsiae in these areas ranged from 0.6 to 12.4%. Isolation and serotyping of these rickettsiae remain to be done.

Isolation of SFG rickettsiae from Ixodes dammini from Shelter Island, New York, suggests that this tick, all stages of which readily attack man, may be involved as a vector of spotted fever rickettsiae in the eastern parts of the U.S.

A SFG rickettsia has been detected in up to 35% of Amblyomma americanum collected in several southern and southeastern states. The fact that residents bitten by infected ticks did not develop illness suggests that this rickettsia is nonpathogenic for man. Laboratory investigations (see 1976-77 Project No. Z01 AI 00065-04 RML) with this agent have now been concluded and indicate that this organism is related to, but quite distinct from, any hitherto described SFG agent. Differences include various morphologic (electron microscopy), physiologic (behavior in laboratory animals), biochemical (protein composition as determined by SDS-polyacrylamide gel electrophoresis) as well as serologic microimmunofluorescence (micro-IF) test characteristics.

The final results of the cooperative study with the Harvard School of Public Health on spotted fever on Cape Cod, Massachusetts, indicate that R. montana does not play a role in the etiology of this disease on Cape Cod. In 1977-78, a total of 7,302 D. variabilis were tested. Seventy-nine (1%) were found to be infected with SFG rickettsiae. Of these, 43 isolates were made and identified. Thirty-nine were R. montana, one was R. rickettsii, and 3 were of an unclassified serotype. The R. rickettsii isolate came from a tick removed from a patient with spotted fever. Of 117 dogs with antibodies to SFG rickettsiae, 65 (56%) had at least four-fold or higher titers to R. montana than to R. rickettsii. Nineteen (16%) had greater titers to R. rickettsii and 33 (28%) had equivalent titers to both serotypes.

A survey of viral, rickettsial, and protozoan agents in ticks from Switzerland revealed a hitherto undescribed SFG rickettsia ("Swiss agent") in up to 11.7% of adult I. ricinus. Infection in ticks was found to be generalized with intracellular as well as intranuclear growth, and trans-ovarial passage to 100% of filial ticks. The Swiss agent is nonpathogenic for guinea pigs but in meadow voles (Microtus pennsylvanicus) produces a microscopically detectable infection in the tunica vaginalis. It does not grow in artificial media but propagates well in tissue culture systems including chick embryo fibroblast, Vero, and vole-tissue cells. It kills 100% of chicken embryos 5 to 7 days after yolk sac inoculation. Antigenic relatedness of the Swiss agent to SFG rickettsiae is indicated by direct fluorescent antibody staining. Serologic typing by micro-IF shows that this agent is quite different from R. rickettsii, R. sibirica, R. slovaca, and R. conorii. So far, there is only serologic evidence that the Swiss agent may be pathogenic for man.

Electron microscopic investigations of the Swiss agent in its tick vector, I. ricinus, revealed rickettsiae in early spermatids. This observation induced us to re-evaluate sexual transmission of rickettsiae by tick sperms a phenomenon claimed to be an important means for the dissemination and maintenance of the spotted fever agent, R. rickettsii, in nature. The results of this investigation are not as yet available.

During a tick/rickettsial survey in an area of western Montana where human cases of spotted fever have never occurred, up to 75% of adult D. andersoni were found to be infected with a SFG agent that was limited in its development to the ovarial tissues and occasionally also to certain parts of the Malpighian tubules. The organism has been isolated and appears to be related to SFG rickettsiae. Since it is nonpathogenic for laboratory animals and any of several indigenous rodents tested so far, it is speculated that it is maintained in nature by transovarial passage only and may indeed be responsible for rendering its tick vector refractory to infection with virulent R. rickettsii. Experiments to clarify this question are in progress.

Serologic characterization of rickettsial isolates from ticks taken off residents in western Montana or collected from vegetation (see 1977-78 Project No. ZO1 AI 00065-05 RML) revealed the presence of at least 4 serotypes of SFG rickettsiae, namely R. rickettsii, R. montana, R. rhipicephali, and 369-C - an organism indistinguishable from a rickettsia isolated initially from D. variabilis in Arkansas. In cooperation with staff of RML's Laboratory of Microbial Structure and Function, a long-term project was initiated to determine the antigenic makeup and stability of these serotypes as well as their physiologic behavior in tick and vertebrate hosts. Additional investigations pertain to the susceptibility of various vertebrate hosts indigenous to western Montana and to the question of interference among these serotypes of rickettsiae.

Male guinea pigs are being used by Dr. Kindmark as a model for the development of an early diagnostic procedure for rickettsial diseases in man. Animals infected with a virulent strain of R. rickettsii are bled on day 4 of fever and are examined for rickettsial antigens by direct and indirect immunofluorescent staining of smears prepared from the buffy coat of blood samples, and by immunoadsorbence in combination with electro-immuno-osmophoresis or electrophoresis in agarose containing antibodies to R. rickettsii. The appearance of early specific antibodies is being determined by the indirect immunofluorescence test. The results obtained so far are promising and suggest that these methods may be applicable to an early diagnosis of spotted fever and related rickettsial diseases of man.

Publications:

Loving, S. M., Smith, A. B., DiSalvo, A. F., and Burgdorfer, W.: The distribution and prevalence of spotted fever group rickettsiae in ticks from South Carolina with an epidemiological survey of persons bitten by infected ticks. Am. J. Trop. Med. Hyg. 27: 1255-1260, 1978.

Burgdorfer, W.: The Rocky Mountain wood tick: Beware its bite! Montana Outdoors May/June: 29-31, 1979.

Hayes, S. F. and Burgdorfer, W.: Ultrastructure of Rickettsia rhipicephali, a new member of the spotted fever group rickettsiae in tissues of the host vector Rhipicephalus sanguineus. J. Bacteriol. 137: 605-613, 1979.

In press:

Burgdorfer, W.: The spotted fever-group diseases. In Steele, J. H. (Ed.): CRC Handbook of Zoonoses

Burgdorfer, W.: Rickettsioses. Chapter 34. In Hubbert, McCulloch and Schnurrenberger (Eds.): Supplementum to "Diseases Transmitted from Animals to Man"

Magnarelli, L. A., Anderson, J. F., and Burgdorfer, W.: Rocky Mountain spotted fever in Connecticut: human cases, spotted fever-group rickettsiae in ticks, and antibodies in mammals. Am. J. Epidemiol.

Linnemann, C. C., Jr., Schaeffer, A. E., Burgdorfer, W., Hutchinson, L., and Philip, R. N.: Rocky Mountain spotted fever in Clermont County, Ohio. II. Distribution of population and infected ticks in an endemic area. Am. J. Epidemiol.

Aeschlimann, A., Burgdorfer, W., Matile, H., Peter, O., and Wyler, R.: Aspects nouveaux du rôle de vecteur joué par Ixodes ricinus L. en Suisse. Note préliminaire. Acta Trop.

Burgdorfer, W., Aeschlimann, A., Peter, O., Hayes, S. F., and Philip, R. N.: Ixodes ricinus: vector of a hitherto undescribed spotted fever group agent in Switzerland. Preliminary note. Acta Trop.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00063-09 EB

PERIOD COVERED: October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Immune Responses to Legionnaires' Bacterium

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. A. Ormsbee	Scientist Director (retired)	
	M. G. Peacock	Microbiologist	EB NIAID
OTHER:	R. N. Philip	Medical Director	EB NIAID
	E. A. Casper	Nurse Director	EB NIAID
	J. C. Williams	Biochemist	LMSF NIAID

COOPERATING UNITS (if any)

Dr. Paul Fiset, Dept. of Microbiology, University of Maryland School of Medicine, Baltimore

LAB/BRANCH

Epidemiology Branch (RML), Hamilton, MT 59840

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.1	1.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study immune responses in man and experimental animals to natural and experimental infections of Legionella pneumophila, the causative agent of Legionnaires' disease. This past year, we have compared the changes in pathogenicity, limits of infectivity in guinea pigs, behavior in tissue culture systems, and other variations of four strains of L. pneumophila when cultivated from embryonated hens' eggs and after numerous further passages on artificial growth media.

PHS-6040
(Rev. 10-76)

Project Description:

Isolates of Legionnaires' disease bacterium (LDB) from fatal cases of Legionnaires' disease were furnished by Dr. McDade, Center for Disease Control, (strain LAC), Mr. Sideman, Pennsylvania Department of Health, (strain LAP), and Ms. Janice Jernigan, Washington State Department of Health, (strain LAW). A fourth strain (LAB) was isolated from cord blood of a healthy infant.

Isolates of Legionella pneumophila were cultured in yolk sacs of embryonated hens' eggs and then serially passed on Mueller Hinton (MH) agar supplemented with hemoglobin and cysteine HCl for 9 and 12 passages. Seeds prepared from yolk sacs of infected chick embryos and from enriched MH agar were titrated in guinea pigs and on primary chicken embryo cell cultures. The parameters of infectivity and changes in pathogenicity of the four strains of L. pneumophila on continued passage on MH media were compared. The LAC, LAP and LAB strains of LDB lost their ability to kill guinea pigs when passed numerous times on MH agar. The LAW strain of LDB had been passed numerous times on artificial media before being received at this laboratory. This strain was never lethal for guinea pigs, even at very high concentrations of viable organisms. The plaquing ability of LAB, LAC and LAP strains was also greatly diminished after passage on MH medium. The LAW strain plaque-forming titers remained unaltered after 12 passages on the artificial media.

Dr. Ormsbee retired in January 1979, but this project will be continued by Mr. Peacock under general supervision by Dr. Philip. Current investigations are underway to determine whether the changes in pathogenicity observed after continued passage of L. pneumophila on artificial media are stable variations and whether they are also accompanied by immunologic changes. Guinea pigs and laboratory mice will be used in challenge studies and indirect immunofluorescence tests of convalescent sera to monitor immunologic responses. We are also looking for biochemical markers of the MH-passed strains to explain the changes noted. Future plans include continued investigations on improvement of antigens used in the micro-immunofluorescence diagnostic test for Legionnaires' disease. The nature of cytopathogenicity in cell cultures will also be studied.

Publications:

Ormsbee, R., Peacock, M., Philip, R., Casper, E., Plorde, J., Gabre-Kidan, T., and Wright, L.: Antigenic relationships between the typhus and spotted fever groups of rickettsiae. Am. J. Epidemiol. 108: 53-59, 1978.

Ormsbee, R. A., Peacock, M. G., Lattimer, G. L., Page, L. A., and Fiset, P.: Legionnaires disease: antigenic peculiarities, strain differences, and antibiotic sensitivities of the agent. J. Infect. Dis. 138: 260-264, 1978.

Peacock, M. G., Fiset, P., Ormsbee, R. A., and Wisseman, C. L., Jr.: Antibody response in man following a small intradermal inoculation with Coxiella burnetii phase I vaccine. Acta Virol. 23, 73-81, 1979.

In press:

Kimbrough, R. C., Ormsbee, R., Peacock, M., Rogers, W. R., Bennetts, R. W., Raff, J., Krause, A., and Gardner, C.: Q fever endocarditis in the United States. J.A.M.A.

Ormsbee, R. A.: A review. Studies in Pyroplasma hominis ("Spotted fever" or "Tick fever") of the Rocky Mountains by Louis B. Wilson and William M. Chowning. Rev. Infect. Dis.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00065-06 LMSF-EB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Antigens and Classification of the Rickettsiae

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. L. Anacker	Research Microbiologist	LMSF NIAID
	R. N. Philip	Medical Director	EB NIAID
OTHER:	W. Burgdorfer	Res. Entomologist (Med.)	EB NIAID
	E. A. Casper	Nurse Director	EB NIAID
	T. F. McCaul	NIH Visiting Fellow	LMSF NIAID
	L. A. Thomas	Research Microbiologist	EB NIAID

COOPERATING UNITS (if any)
Dr. Robert Lane, California Department of Health, Berkeley, CA

LAB/BRANCH Laboratory of Microbial Structure and Function and Epidemiology Branch (RML), Hamilton, MT 59840

SECTION
Rickettsial Diseases Section and Epidemiology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.6 (1.9 - EB)	2.7 (1.0 - EB)	2.9 (0.9 - EB)

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

See page 28-13 of Annual Report of Laboratory of Microbial Structure and Function.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 AI 00067-14 FB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Tularemia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. F. Bell

COOPERATING UNITS (if any)

LAB/BRANCH
Rocky Mountain Laboratory

SECTION
Medical Zoology and Zoonotic Diseases Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Project terminated

Bell, J. F., Wikel, S. K., Hawkins, W. W., and Owen, C. R.: Enigmatic resistance of sheep (Ovis aries) to infection by virulent Francisella tularensis. Can. J. Comp. Med. 42: 310-315, 1978.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00068-18 EB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Rabies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. F. Bell

COOPERATING UNITS (if any)

LAB/BRANCH
Rocky Mountain Laboratory

SECTION
Medical Zoology and Zoonotic Diseases Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Project terminated

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00069-48 EB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Systematics and Vector Relationships of Certain Parasitic Arthropods

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. M. Clifford	Scientist Director	EB NIAID
OTHER:	J. E. Keirans	Res. Entomologist (Med.)	EB NIAID
	C. E. Yunker	Scientist Director	EB NIAID

COOPERATING UNITS (if any) Drs. H. Hoogstraal, NAMRU-3; D. Sonenshine and P. Homsher, Old Dominion Univ.; A. J. Spielman, Harvard School of Pub. Hlth; D. Stiller, USDA: F. S. Lukoschus, Catholic Univ., Netherlands; W. Buttiker, Ciba-Geigy, Riyadh, Saudi Arabia; Miss Jane Walker, Div. of Vet. Services, Onderstepoort.

LAB/BRANCH
Epidemiology Branch (RML), Hamilton, MT 59840

SECTION
Arthropod-Borne Diseases Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.4	2.0	2.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The activities of this project currently comprise four main functions: 1) Identification of ticks received from various individuals and government agencies throughout the world. Only one other institution in the world is capable of performing this service. 2) Systematic study of certain groups of parasitic arthropods. The foremost tool in systematic studies, the scanning electron microscope, has greatly aided in elucidating taxonomic concepts in acarines actually or potentially involved in transmission of disease agents. 3) Final editing and storage of all RML and NAMRU-3 (Cairo) tick data in the Smithsonian data retrieval system. 4) Colonization of medically important arthropod vectors, which are furnished to outside investigators.

PHS-6040
(Rev. 10-76)

Project Description:

The primary objective of this project is the systematic treatment, including taxonomy and classification, biology, ecology, and colonization, of parasitic arthropods of known or potential medical importance.

Argasid ticks. Our studies on the genus Argas have continued with the emphasis being placed on the morphology of Haller's organ. During the past year, it was decided to expand the investigations to include more species of each subgenus. This will necessitate publishing three separate manuscripts, i.e., one on the subgenus Argas, another on Persicargas and Microargas, and a last paper on Carios, Chiropterargas, Secretargas, and Ogadenus. As we previously stated, we hope to correlate the morphology of this organ with the host-specificity and other behavioral characteristics of these ticks.

Significant progress was made on our continuing study of the Ornithodoros capensis complex. We have redescribed O. amblus that feeds mainly on marine birds in Peru and have prepared scanning electron microscope (SEM) photos of O. muesebecki for possible redescription during the next year. Clarification of the status of these two ticks is deemed important because they frequently bite humans who invade their domain causing severe reactions. Also, viruses have been isolated from O. amblus and O. muesebecki that may cause illness in the humans and birds the ticks feed on.

We are completing descriptions of O. darwini and O. galapagensis, ectoparasites of land and marine iguanas and lava lizards in the Galapagos Islands. It is very uncommon to find members of the tick genus Ornithodoros parasitic upon reptiles. Two species of intraerythrocytic hemogregarines are found in the blood of marine iguanas and lava lizards and ticks are probable vectors.

Ixodid ticks. A manual to allow easier identification of ticks of the genus Dermacentor is still in preparation. This manual is to be similar to our work on the genus Ixodes and will be amply illustrated with SEM photos.

A study was initiated to clarify the status of an Ixodes species of the angustus complex that occurs in Spearfish Canyon in South Dakota. The ticks in this canyon are particularly important because Powassan virus, a disease of humans, has been isolated in this area from Ixodes ticks.

Our continuing investigations of the medically and agriculturally important rhipicephalid species of Africa is progressing nicely. A manuscript clarifying the status of Rhipicephalus kochi is being written and another on R. tricuspis and the R. appendiculatus group has been started. Hundreds of SEM photographs have been taken of African ticks of this genus.

We also hope to initiate a study of the medically important R. sanguineus complex. Hopefully, this will be accomplished by acquiring a predoctoral level student who is familiar with this problem. We are attempting to obtain funding for Mr. Rupert Pegram of Brunel University, Uxbridge, England, who is working on a Master's thesis on the genus Rhipicephalus in Ethiopia and Somali and would have a fine background for our project.

Mite studies. Manuscripts describing clinical mange in the brown bear and in laboratory primates, as well as the macronyssid fauna of Surinam, have been submitted for publication. The parasitic Acari of Saudi Arabia are being studied with the aim of providing a fauna list and key for public health workers. Numerous identifications of parasites, some involved in human or animal illness, were made. Institutions requiring identifications were: Center for Disease Control (Atlanta), U.S. Naval Medical Research Institute (Bethesda), Eppley Institute for Cancer Research (Omaha), University of South Dakota (Brookings), Canada Department of Agriculture (Lethbridge), Cambridge University (Cambridge), Panamerican Health Organization (Panama), Sudanese Veterinary Research Administration (Khartoum), and University of Cairo (Egypt).

RML-NAMRU-3 computer data. Final editing of the master list of the data on the RML and NAMRU-3 tick collection has been completed and all the errors noted have been corrected. We are currently awaiting Dr. Gautier of the Smithsonian to complete his work on the data file. This is supposed to be completed by September of 1979. Following this a mini file will be constructed to save money in querying the file and we will be able to begin using the information for the first time.

In the meantime, new records and corrections are rapidly accumulating. We have asked for some technical help to alleviate this situation, but as yet no solution has been found. It is imperative that the continuity of entry of the new data and corrections be maintained.

Nuttall Collection. Results of a year's research on the G.H.F. Nuttall tick collection are now being compiled. This is the third largest tick collection ever assembled (behind those of RML and H. Hoogstraal) and comprises roughly 3,900 collections ranging from one to several hundred specimens and contains material from all zoogeographic areas of the world. The collection contains over 100 type species, many in need of taxonomic clarification, that are known vectors of human and animal disease agents. A book cataloging this collection is being prepared under the aegis of the British Museum (NH), the depository for this collection.

In conjunction with the above project, a second important tick collection, that of Nathanial C. Rothschild of the famed banking family, was redetermined and updated. He deposited 197 tick collections with Professor

Nuttall and, between 1911 and 1922, deposited an additional 155 collections in the British Museum (NH). A manuscript on this work has just been completed.

In 1976 we petitioned the International Commission of Zoological Nomenclature to use its plenary powers to conserve the name Dermacentor andersoni, one of the world's most important ticks in terms of medical importance. The Commission has just delivered a favorable verdict. The name D. venustus has been suppressed and can no longer be used for the Rocky Mountain wood tick.

Service oriented activities. Several thousand ticks were identified for various laboratories throughout the world. Also, over 222,000 ticks were furnished to investigators at the RML and elsewhere for experimental studies on ticks and tickborne diseases. The ticks submitted to us for identification continue to be an excellent source of new tick material for our own research interests.

The future course of this project will emphasize the use of the SEM to clarify taxonomic problems among various genera of ticks. Special attention will be devoted to the groups of ticks from which we are isolating viruses and other disease agents. We will also stress the work on groups of ticks like the African rhipicephalids that are proven vectors of diseases of humans and other animals. We plan to produce additional practical guides to the identification of ticks that are amply illustrated with SEM photos.

Publications:

Keirans, J. E. and Clifford, C. M.: The genus Ixodes in the United States: a scanning electron microscope study and key to the adults. J. Med. Entomol. Supplement No. 2: 1-149, 1978.

Clifford, C. M., Hoogstraal, H., Keirans, J. E., Rice, R.C.A., and Dale, W. E.: Observations on the subgenus Argas (Ixodoidea: Argasidae: Argas). 14. Identity and biological observations of Argas (A.) cucumerinus from Peruvian seaside cliffs and a summary of the status of the subgenus in the Neotropical Faunal Region. J. Med. Entomol. 15: 57-73, 1978.

Clifford, C. M., Keirans, J. E., Hoogstraal, H., and Morel, P. C.: Observations on the subgenus Argas (Ixodoidea: Argasidae: Argas). 15. Identity of Argas (A.) magnus from Ecuador and Colombia. J. Med. Entomol. 15: 157-165, 1979.

Siuda, K., Hoogstraal, H., Clifford, C. M., and Wassaf, H. Y.: Observations on the subgenus Argas (Ixodoidea: Argasidae: Argas). 17. Argas (A.) polonicus sp. n. parasitizing domestic pigeons in Krakow, Poland. J. Parasitol. 65: 170-181, 1979.

Keirans, J. E., Hoogstraal, H., and Clifford, C. M.: Observations on the subgenus Argas (Ixodoidea: Argasidae: Argas). 16. Argas (A.) moreli, new species, and keys to Neotropical species of the subgenus. J. Med. Entomol. 15: 246-252, 1979.

Spielman, A., Clifford, C. M., Piesman, J., and Corwin, D.: Human babesiosis on Nantucket Island, USA: description of the vector, Ixodes (Ixodes) dammini, n. sp. (Acarina: Ixodidae). J. Med. Entomol. 15: 218-234, 1979.

In press:

Stark, H. E., Inlao, I., Clifford, C. M., Keirans, J. E., and Lakshana, P.: A preliminary checklist of the ticks of Thailand. Appl. Sci. Res. Corp J. Thailand

Keirans, J. E., Clifford, C. M., and Yunker, C. E.: Rocky Mountain Laboratory reference collection of ticks and parasitic mites (Acari). A Guide to the Parasite Collections of the World.

Corwin, D., Clifford, C. M., and Keirans, J. E.: An improved method for cleaning and preparing ticks for examination with the scanning electron microscope. J. Med. Entomol.

Hoogstraal, H., Clifford, C. M., and Keirans, J. E.: The Ornithodoros (Alectorobius) capensis group (Acarina: Ixodoidea: Argasidae) of the Palearctic and Oriental regions. O. (A.) coniceps: identity, bird and mammal hosts, virus infections, and distribution in Europe, Africa, and Asia. J. Parasitol.

Hoogstraal, H., Clifford, C. M., Keirans, J. E., and Wassef, H. Y.: Recent developments in biomedical knowledge of Argas ticks (Ixodoidea: Argasidae). Proc. 5th Intl. Congress of Acarology

Appendix 1. PL-480 Project 03-063N. Epidemiological and Biological Aspects of Tick-Borne Infections in Egypt and Elsewhere, NAMRU-3, Cairo, Egypt

Annual Funding: $123,000

The work done in the Bibliography Section at NAMRU-3 continues to provide the scientific world with excellent reference material. Volume 5, Part II, has been published and includes over 2500 references for 1974, 1975 and 1976 and 2400 additions to Volumes 1-5, Part I. Attention is now being directed toward completing the Annotated Bibliography of Tickborne Viruses. The section on the Bunyaviridae should be published in 1979. Numerous (250) translations were completed and several species of ticks were expertly illustrated.

The serological survey for tickborne viruses of potential public health importance also produced some excellent results. Sera from domestic animals were tested for the presence of four arboviruses, i.e., Dugbe, Crimean-Congo hemorrhagic fever, Bhanja and Tettnang virus. Antibodies to all four viruses were found in Egypt.

Large volumes of human sera were also tested in this program for the presence of antibodies to Rift Valley Fever (RVF) - a disease which infected large numbers of people in Egypt in 1977 and 1978. About 3000 sera collected in 1974, 1975 and 1976 were negative for antibodies, which helps to establish that RVF was not present in Egypt prior to the recent outbreak. Also, tests of human sera collected in 1977 and 1978 demonstrated the presence of RVF antibodies in 19% of the samples.

During the next year, a larger number of tickborne viruses will be tested in human sera samples from various parts of Egypt. Further studies on RVF fever, such as determining the vector of this virus in Egypt and where the virus overwinters will be undertaken during the next year. As mentioned above, the work being conducted at Cairo University is in preliminary stages. This is partly due to the fact that although funds were received for a full year, approval was granted near the first of the year.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 AI 00079-19 EB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

The Encephalitides

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: L. A. Thomas

COOPERATING UNITS (if any)

LAB/BRANCH
Epidemiology Branch (RML), Hamilton, MT 59840

SECTION
Epidemiology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	--

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Project terminated

In press:

Thomas, L. A., Patzer, E. R., Cory, J. C., and Coe, J. E.: Antibody development in garter snakes (Thamnophis spp.) experimentally infected with Western equine encephalitis virus. Am. J. Trop. Med. Hyg.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00080-12 EB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Tickborne Disease Agents

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. E. Yunker	Scientist Director	EB NIAID
OTHER:	C. M. Clifford	Scientist Director	EB NIAID
	J. E. Keirans	Res. Entomologist (Med.)	EB NIAID
	L. A. Thomas	Research Microbiologist	EB NIAID

COOPERATING UNITS (if any) Drs. J. Casals, YARU, New Haven; R. Gresbrink, Oregon State Bd. Hlth., Portland; H. Hoogstraal, NAMRU-3, Cairo, Egypt; B. Easterday, Dept. of Vet. Sci., Univ. Wisconsin, Madison; K. King, Patuxent Wildlife Res. Ctr.; E. Easton, S. Dakota State Univ.; D. G. McKercher, Univ. Calif., Davis.

LAB/BRANCH
Epidemiology Branch (RML), Hamilton, MT 59840

SECTION
Arthropod-Borne Diseases Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.8	0.9	1.9

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In this project the incidence, distribution and ecology of tick-borne disease agents, arboviruses and rickettsiae, are studied. These pathogens are isolated in tissue cultures or animals from field-collected materials and are serologically identified. Viruses, if new, are physicochemically and biologically characterized and taxonomically classified. Of particular importance is the delineation of vector-relationships and human disease potential of the increasing number of tick-borne viruses in the United States.

PHS-6040
(Rev. 10-76)

Project Description:

Over 1100 ticks, as well as 166 blood samples from humans, rodents and birds, collected in seven states, mostly by public health or wildlife disease investigators, were tested for pathogens. These tests resulted in 102 isolations of viruses and 22 of rickettsiae. All blood samples proved negative for pathogens.

Among the agents identified were 30 isolates of Soldado virus from Ornithodoros capensis ticks in Texas (infection rate [IR], 10.6%) and 25 of Hughes virus from O. denmarki in Florida (IR, 14%). Both tick species are parasites of migratory seabirds and their viruses are distantly related members of an unclassified taxon of viruses currently termed the Hughes virus serogroup. Some Hughes group agents have been implicated in febrile disease of humans and in seabird die-offs. Fifteen additional isolations of Sapphire II virus (also of the Hughes serogroup) were made this year from South Dakota populations of Argas cooleyi, a parasite of migratory passerine birds. These same ticks yielded 16 strains of Sixgun City virus, a Reovirus of the genus Orbivirus. For each virus, the infection rate was calculated to be about 3%. Five viral isolates from these ticks remain unidentified.

A new virus of the Uukuniemi serogroup (Bunyaviridae), which we discovered last year in seabird parasites, Ixodes uriae, on St. Paul I., Alaska, was physicochemically and biologically characterized in preparation for publication. Its virion is between 100 and 220 nm in diameter and contains RNA as well as a surrounding lipid-containing envelope. While fairly resistant to thermal inactivation, the virus is readily denatured by exposure to acid pH. It is highly pathogenic for newborn mice by intracerebral and peripheral routes of inoculation, but causes illness and death in weanlings only by intracerebral route. It fails to plaque in Vero cells, but does so in Xenopus cells regardless of exposure of the virus to neutralizing antibody. No virus growth was seen in mosquito or tick cell cultures.

This year, we isolated an unidentified virus from Dermacentor occidentalis collected in two counties of Oregon. The tick, a man-biting species, has previously been shown to harbor Colorado tick fever virus, as well as rickettsiae of the spotted fever group, but the existence of this serologically unique virus was unsuspected. Fourteen isolations of the new agent were made in Xenopus cells, but none were obtained in Vero cells or by injection of newborn mice. The virus, while not pathogenic for newborn white mice or hamsters, does cause illness in meadow voles. It will propagate in L cells, pig kidney cells, and tick tissue cultures and is presently being characterized with regard to its physicochemical properties. At the request of California researchers, a virus isolated from aborting cattle and their tick parasites, O. coriaceus, was also partially characterized.

The rickettsiae were isolated in Vero and Xenopus cell cultures from three species of ticks known to bite man: I. pacificus and D. occidentalis (from Oregon) and D. variabilis (from South Dakota). Two rickettsial entities, as determined by microimmunofluorescence test, were involved in 16 of the isolations. Both are members of the spotted fever group and have previously been recorded by us from the respective tick species. Six additional isolations remain to be identified.

Last year, in attempts to increase the sensitivity of our assay procedures for isolation and identification of tickborne pathogens of low virulence and antigenicity, we extensively tested an amphibian cell line derived from Xenopus laevis. As noted above, this cell line of poikilothermic origin was the only system capable of detecting the unidentified virus from D. occidentalis. Hence, it may prove useful in the isolation of temperature sensitive variants. In addition, it alone accounted for all but three of the Soldado virus isolations mentioned above, and is obviously superior to Vero cells and newborn mice for isolation of this tickborne virus. Its advantages in the isolation of spotted fever group rickettsiae have previously been noted. Also, these cells have proven useful in the production of viral antigens for microimmunofluorescence tests.

We plan to continue this project as before with the assistance in the field of collaborating investigators. Geographical records of tickborne viruses in various vectors and in flyways of migratory birds enable us to predict the distribution and prevalence of these pathogens. For example, the many isolations obtained this year of Hughes group viruses support our earlier evidence of vector-specificity for these viruses. From this we may infer that their distributional patterns are primarily those of the vector. Also, the high infection rates for the viruses under study suggests a dual role of the tick as vector as well as maintenance host. Arboviruses transmitted by mosquitoes and sandflies are relatively well studied in comparison to those that are tickborne. Thus, this project helps to fill an obvious void in our understanding of the natural history of arboviruses.

Publications:

In press:

Yunker, C. E.: Current research on transmission of disease by Acari. Proc. Symp. Vth Intl. Congress of Acarology, East Lansing, MI, Aug. 6-12, 1978

Yunker, C. E., Thomas, L. A., and Cory, J.: Phenetic relationships of viruses of the Hughes serological group. Proc. 2nd Int. Symp. on Arctic Arboviruses, Mt. Gabriel, Que., Canada, May 26-28, 1977. New York, Academic Press.

Yunker, C. E., Clifford, C. M., Keirans, J. E., Thomas, L. A., and Rice, R.C.A.: Isolation and characterization of a new tickborne virus of the Upolu serogroup, new serogroup, from coastal Texas. J. Med. Entomol.

Clifford, C. M.: Tick-borne viruses of seabirds. Proc. 2nd Int. Symp. on Arctic Arboviruses, Mt. Gabriel, Que., Canada, May 26-28, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00081-18 EB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
The Epidemiology of Human Infections of Special Interest

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. N. Philip	Medical Director	EB NIAID
OTHER:	E. A. Casper	Nurse Director	EB NIAID
	L. A. Thomas	Research Microbiologist	EB NIAID
	W. Burgdorfer	Res. Entomologist (Med.)	EB NIAID
	R. L. Anacker	Research Microbiologist	LMSF NIAID

COOPERATING UNITS (if any) Dr. Karim Hechemy, New York State Dept. of Health, Albany; Dr. Richard Emmons, Viral and Rickettsial Disease Laboratory, California Dept. of Health, Berkeley; Dr. Jorge Benach, New York State Dept. of Health, Stony Brook; Dr. Catharine Wilfert, Duke Univ. Medical Center, Durham, NC.

LAB/BRANCH
Epidemiology Branch (RML), Hamilton, MT 59840

SECTION
Epidemiology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.4	1.6	0.8

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☒ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project emphasizes epidemiologic investigations of infectious diseases of particular interest to the research program of this laboratory. Current investigations include studies of the etiologic relationship of various spotted fever-group serotypes to human illness, and improvement in laboratory methods for diagnosis of rickettsial infections, particularly Rocky Mountain spotted fever.

PHS-6040
(Rev. 10-76)

Project Description:

Etiologic role of spotted fever group (SFG) agents to human infection. A study to determine the importance of SFG organisms other than Rickettsia rickettsii as a cause of human infection was continued in collaboration with Dr. Burgdorfer's staff. Attached Dermacentor andersoni, taken from Bitterroot Valley residents and shown to be infected with SFG rickettsiae by hemolymph test, were inoculated into microtus and Vero cell cultures. Nine isolates were obtained in cell culture and/or microtus from 33 infected ticks that had been stored for 1 to 2 years at 65°C. The isolates included 4 strains of R. rickettsii, 2 of R. rhipicephali, 2 of 369-C, and one of R. montana. In addition, 8 ticks gave rise to antibodies in microtus which typed by microimmunofluorescence (micro-IF) as R. rhipicephali (1) and 369-C (7). Thus, the infecting strain was identified in 17 of the 33 ticks. Aside from 3 cases of Rocky Mountain spotted fever (RMSF) from whom R. rickettsii-infected ticks were obtained, none of the persons with tick bite had clear-cut clinical or serologic evidence of associated infection.

In collaboration with Dr. Benach, a serologic study was conducted among permanent residents of Shelter Island located at the eastern tip of Long Island. In recent years, this locale has been heavily infested with D. variabilis and Ixodes dammini due in part to curtailment in clearing of underbrush by burning. Hemolymph tests revealed rickettsia-like organisms in 14% of D. variabilis surveyed one year ago. A serosurvey of Shelter Island residents for micro-IF antibodies to various members of the SFG in June 1978 revealed a significantly higher prevalence of low level reactivity than was found among residents of New York City and Long Island. The same population cohort of Shelter Island residents was retested in October 1978. An amazingly high 14% of 103 participants showed serologic conversion by micro-IF against rickettsiae of the SFG during the 4-month interval, particularly against R. rickettsii and the 369-C agent. Yet there was no clinical evidence of RMSF in this population group. The etiology of the serologic reactivity has not been determined, but whatever the cause, it was not associated with a clinical response.

Serologic tests. The investigation of the suitability of the indirect hemagglutination test for the detection of spotted fever antibody in man and several kinds of laboratory animals (Anacker) was completed during the past year. Antibody in sera from human patients was detected, with human group "O" erythrocytes sensitized with an alkali digest of purified R. rickettsii as antigen, as early as 3 days and as late as 3 years after onset of illness. Both human IgG and IgM antibodies participated in the reaction but IgM antibodies were much more efficient. Antibody which agglutinated both sensitized fresh and glutaraldehyde-fixed sheep erythrocytes was found in rabbit sera at all intervals tested (10-59 days postinfection). Antibody which agglutinated fresh but not

glutaraldehyde-fixed sheep erythrocytes was detected in guinea pigs 7, 14, and 28 days postinfection. Antibody was found in mice inoculated with a high dose of R. rickettsii but not with a low dose. This hemagglutination test should be of value for serologic diagnosis of spotted fever infection of humans and for laboratory studies of spotted fever infections of common laboratory animals.

We are assisting Dr. Hechemy in the development of a latex agglutination test for diagnosis of RMSF. Latex particles complexed with the alkali digest of purified R. rickettsii from a stable suspension which can be stored for many months. This suspension is rapidly and specifically agglutinated by sera from most spotted fever patients, particularly those with IgM antibodies. Presently the latex agglutination test is being compared with the micro-IF and complement-fixation tests for detection of antibody in sera from patients from different regions of the United States. The latex agglutination test may prove to be the simplest and most economical of all tests for the routine serodiagnosis of spotted fever.

In collaboration with Dr. Emmons, we are investigating the etiologic relationships of SFG agents with RMSF-like illnesses associated particularly with D. occidentalis in the coastal areas of California outside the range of D. andersoni. In one instance, paired acute and convalescent serum samples obtained from a mild RMSF-like illness associated with tick bite acquired in coastal northern California were shown to have a modest micro-IF antibody titer rise to various SFG agents including isolates obtained from D. occidentalis in that area (see Project No. Z01 AI 00065-06 LMSF-EB).

The attributes of an enzyme-linked immunosorbent assay (ELISA) test and a solid-phase immunofluorescence procedure (FIAX) for quantitating rickettsial antibody are being defined. Current information indicates that both test systems are about 4- to 8-fold more sensitive than our micro-IF test for detection of antibodies against R. rickettsii in convalescent sera from patients with RMSF. Comparably low concentrations of the same soluble rickettsial antigen are optimal for both test systems. For this reason, we are optimistic that these procedures can be used for early detection of antigen in body fluids of patients with RMSF. These studies will begin shortly and will occupy much of the activity in this project during the coming year. We will also be involved in providing serologic support for an epidemiologic study of RMSF in 2 counties in North Carolina (Wilfert). Our role will be to obtain ancillary information on the etiologic relationship of various SFG agents to human illness. Similar investigations will be continued in collaboration with the California State Viral and Rickettsial Disease Laboratory (Emmons).

Publications:

Hechemy, K. E., Stevens, R. W., Sasowski, S., Michaelson, E. E., Casper, E. A., and Philip, R. N.: Discrepancies in Weil-Felix and microimmunofluorescence test results for Rocky Mountain spotted fever. J. Clin. Microbiol. 9: 292-293, 1979.

Philip, R. N., Casper, E. A., and Burgdorfer, W.: Current knowledge of the distribution of serotypes of spotted fever-group rickettsiae in the United States as determined by microimmunofluorescence. In: Proceedings VII International Congress of Infectious and Parasitic Diseases, Varna, Bulgaria, Oct. 2-6, 1978, 1978, pp. 500-509.

In press:

Philip, R. N.: Scrub typhus. In Steele, J. H. (Ed.): CRC Handbook of Zoonoses

Philip, R. N.: Typhus group rickettsiae. In Steele, J. H. (Ed.): CRC Handbook of Zoonoses

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00082-18 EB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Relation of Viruses to the Genesis of Chronic Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: W. J. Hadlow Res. Veterinarian (Path.) EB NIAID

COOPERATING UNITS (if any)
Dr. S. B. Prusiner, Dept. of Neurology, University of California, San Francisco

LAB/BRANCH
Epidemiology Branch (RML), Hamilton, MT 59840

SECTION
Histopathology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.8	1.0	1.8

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is mainly concerned with characterizing several naturally occurring slow viral diseases of domestic animals that resemble important, etiologically obscure diseases of man. Two diseases receive most attention: scrapie of sheep and goats and Aleutian disease of mink. Their clinico-pathologic, virologic, and immunologic features are studied to provide an understanding of their pathogenesis and natural history. Information from such studies will help define the unusual host-virus relationships that result in protracted disease in man and animals.

PHS-6040
(Rev. 10-76)

Project Description:

This project is concerned with learning more about the unusual host-virus relationships that give rise to slowly evolving diseases after a long incubation period. The main diseases studied are scrapie of sheep and goats and Aleutian disease of mink. Because the primary objective of the research is the study of the disease process in each infection, most attention is directed to obtaining information on the temporal replication of the causative virus and on the cellular and humoral host responses in relation to the natural history of the disease. Less attention is directed to the properties of the causative viruses.

Because of an administrative directive to do so, all new work in this project was discontinued about mid April 1978. Moreover, the project has since been stripped of all its supporting personnel but one—a histotechnologist. Thus, even work in progress at the time the directive was issued has suffered severely—valuable observations on experimental animals could not be made, supporting laboratory studies could not be done, and records could not be kept up-to-date. The main concern of the principal investigator, therefore, has been to salvage what he could from the tattered remnants, and this effort has not been too productive so far.

No new observations on scrapie or Aleutian disease were made since the last annual report. Only a few experiments, mostly virus titrations, remain active. And these will be completed within a year.

As mentioned in last year's report, a pilot study was begun about 4 years ago with specimens of brain from 11 persons dying of Creutzfeldt-Jakob disease (CJD). Guinea pigs and mink inoculated intracerebrally with these specimens have remained free of recognizable neurologic disease after 2 and 4 years, respectively. A presumed neurologic disease that occurred in similarly inoculated mice, mentioned in last year's report, has occurred in some of those in the second passage, but the results are still inconclusive.

The only certain positive results so far were in similarly inoculated African pygmy goats. One of 3 that received one brain specimen and 1 of 4 that received another became affected with a scrapie-like disease about 43 months after intracerebral inoculation. In both, the disease was characterized by scratching of the skin, a depressed attitude, and motor difficulties affecting gait. One goat became extremely depressed, lost weight, and had impaired vision. It was killed about 8 weeks after onset of clinical signs, when it spent much of the time recumbent. The other goat, still under observation, wanders about its pen and scratches continually. Three other goats of this group may now be in the prodromal stage of the experimental disease.

Preliminary histologic examination of the brain and spinal cord of the goat that was killed indicated changes compatible with those of caprine scrapie. Astrocytosis was prominent in the diencephalon, mid brain, and cerebellar cortex, but spongiform change was minimal. A more complete evaluation of these microscopic changes is in progress.

Goats inoculated with the other 9 specimens of brain are still normal, but most have not been under observation long enough to warrant any conclusions about whether they too may become affected.

The occurrence of scrapie-like disease in these goats is of considerable interest vis-a-vis the possible relationship of CJD virus and scrapie virus. But whether it provides any better understanding of the origin and identity of CJD virus is still uncertain. CJD virus and scrapie virus also cause indistinguishable neurologic diseases in the squirrel monkey and the rhesus monkey. In the absence of information about the antigenic relation of these viruses, their similarity or common identity no doubt will not be determined by a study of their experimental host ranges. With both viruses, the pattern of neuropathologic changes seen in experimental hosts is influenced by the passage history of the viruses and by other laboratory manipulations imposed upon them. Even so, the occurrence of an encephalopathy resembling scrapie in a natural host of that disease may be more significant than the occurrence of a similar disease in some aberrant host like the monkey or the hamster. At any rate, this finding in goats inoculated with brain tissue directly from man emphasizes a long-held view of the principal investigator: the susceptibility of all common farm animals to scrapie virus infection should be examined not only to clarify some epidemiologic aspects of scrapie, but also to provide information that may be relevant to determining possible sources of human infection with CJD virus. Hosts with inapparent infections may be more important than those in which overt disease occurs.

Except for continuing observations on animals inoculated with brain suspensions from persons dying of CJD, little more is contemplated. Any further work at RML will be largely contingent on what the principal investigator may be allowed to do with these animals. If nothing else, certainly a second passage in goats should be made. In the coming year, then, time will be spent evaluating the large amount of experimental data already collected. But this can not be done single-handedly. So, only that which is more readily accessible and is more easily coped with will be considered. It is not the intent of the principal investigator to spend much of his time in largely clerical tasks; he will devote most of his time to the evaluation of the large amount of histologic material that still needs to be studied. Given the present constraints imposed on the activities of the principal investigator, this approach seems to be the only productive way to deal with what remains of this project.

Publications:

Eklund, C. M. and Hadlow, W. J.: Animal viral diseases as models of central nervous system disease in man. In Vinken, P. J. and Bruyn, G. W. (Eds.): Handbook of Clinical Neurology, Vol. 34: Infections of the Nervous System, Part II. Amsterdam, New York, North Holland Publishing Co., 1978, pp. 291-305.

Prusiner, S. B., Hadlow, W. J., Eklund, C. M., Race, R. E., and Cochran, S. P.: Sedimentation characteristics of the scrapie agent from murine spleen and brain. Biochemistry 17: 4987-4992, 1978.

Prusiner, S. B., Hadlow, W. J., Garfin, D. E., Cochran, S. P., Baringer, J. R., Race, R. E., and Eklund, C. M.: Partial purification and evidence for multiple molecular forms of the scrapie agent. Biochemistry 17: 4993-4999, 1978.

Prusiner, S. B., Garfin, D. E., Baringer, J. R., Cockran, S. P., Hadlow, W. J., Race, R. E., and Eklund, C. M.: Evidence for multiple molecular forms of the scrapie agent. In Stevens, J. G., Todaro, G. J., and Fox, C. F. (Eds.): Persistent Viruses. ICN-UCLA Symposia on Molecular and Cellular Biology. New York, Academic Press, Inc., 1978, vol. VI, pp. 591-613.

In press:

Prusiner, S. B., Garfin, D. E., Baringer, J. R., Cochran, S. P., Hadlow, W. J., Race, R. E., and Eklund, C. M.: On the partial purification and apparent hydrophobicity of the scrapie agent. In Prusiner, S. B. and Hadlow, W. J. (Eds.): Slow Transmissible Diseases of the Nervous System. New York, Academic Press, Inc.

Hadlow, W. J., Race, R. E., Kennedy, R. C., and Eklund, C. M.: Natural infection of sheep with scrapie virus. In Prusiner, S. B. and Hadlow, W. J. (Eds.): Slow Transmissible Diseases of the Nervous System. New York, Academic Press, Inc.

Hadlow, W. J., Kennedy, R. C., Race, R. E., and Eklund, C. M.: Virologic and neurohistologic findings in dairy goats affected with natural scrapie. Vet. Pathol.

Hadlow, W. J.: Myopathy associated with congenital hydrocephalus in Hereford calves. In Andrews, E. J., Ward, B. C., and Altman, N. A. (Eds.): Spontaneous Animal Models of Human Disease. New York, Academic Press, Inc.

In press: (cont'd)

Eklund, C. M. and Hadlow, W. J.: Slow viral diseases. In Steele, J. H. (Ed.): Handbook of Zoonoses, Section B. Viral Zoonoses. West Palm Beach, CRC Press, Inc.

Hadlow, W. J., Cheville, N. F., and Jellison, W. L.: Occurrence of pox in a northern fur seal on the Pribilof Islands in 1951. J. Wildl. Dis.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 AI 00083-10 EB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Host-Ectoparasite Relationships

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: S. K. Wikel

COOPERATING UNITS (if any)

LAB/BRANCH
Rocky Mountain Laboratory

SECTION
Medical Zoology and Zoonotic Diseases Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Project terminated

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00084-10 EB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Vector-Pathogen Relationships

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. E. Yunker	Scientist Director	EB NIAID
OTHER:	L. A. Thomas	Research Microbiologist	EB NIAID

COOPERATING UNITS (if any) Dr. Kouka S.E. Abdel-Wahab, Al Azhar University, Cairo, Egypt; Dr. Mamdouh Y. Kamel, National Research Center, Cairo, Egypt; Dr. D. Knudson, Yale University, New Haven; Drs. L. Rosen and R. Tesh, University of Hawaii, Honolulu.

LAB/BRANCH
Epidemiology Branch (RML), Hamilton, MT 59840

SECTION
Arthropod-Borne Diseases Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	0.4	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project deals with the interactions of vector-borne microorganisms, arboviruses, rickettsiae and protozoans, and cells of haematophagus arthropods, mainly ticks. Model in vitro systems are developed and applied to studies of pathogen host-range, growth dynamics, persistence and interference. After prolonged growth in these systems, pathogens are examined for changes in invasiveness, virulence, or antigenic characteristics. By contract with collaborative laboratories overseas, we are studying critical factors influencing survival of ticks and of pathogens within ticks from a biochemical, histochemical, and physiological standpoint.

PHS-6040
(Rev. 10-76)

Project Description:

A study of transovarial transmission of Flaviviruses by ticks begun last year was continued. Soviet workers have identified DNA transcripts of Bhanja virus RNA in eggs of Hyalomma ticks. A model system of Langat virus in Dermacentor ticks was devised in which virus was recovered in all F_1 stages of female ticks inoculated parenterally and a small percentage of F_2 eggs and larvae. All F_1 stages transmitted virus by bite to laboratory hosts. Virus could not be detected in subsequent F_2 stages, whether fed or unfed. Thus, proviral DNA may not be involved in this particular system. Additional transovarial transmission experiments with Quaranfil virus in Argas arboreus and Colorado tick fever (CTF) virus are in progress.

Carrier cultures of CTF virus were established in Xenopus cells grown at 27°C. After 50 passages (350 days) apparently normal cell cultures continue to yield low titers of CTF virus. Previously we observed a diminished pathogenicity for mice when the virus underwent prolonged growth in mosquito cells incubated at 27°C. However, in the Xenopus cells this virus retains its pathogenicity. This implies that growth at reduced temperature of incubation alone is not a factor in attenuation of this virus in arthropod cells.

The attenuated strain of CTF virus, which we developed by its prolonged passage in arthropod cells, as well as a virulent strain, have been sent to Yale University where they will be examined from a molecular standpoint, with a view toward understanding the genetic basis for Orbivirus virulence.

The application of mosquito cell cultures to the detection of dengue viruses was studied. Isolation and identification of these Flaviviruses in traditional systems is a slow and difficult process and is often uncertain. Mosquitos inoculated in Hawaii with an unspecified number of 4 dengue serotypes were coded, frozen and shipped to RML, where they were ground and inoculated into Aedes albopictus cells. Virus was detected in all but 3 of 24 mosquitos. Sixteen of the isolates were recognized by the appearance of syncitia 4 to 6 days after inoculation and the remainder by microimmunofluorescence to dengue-2 immune serum. Two of the isolates that were definitely missed were of the dengue-3 serotype (which grows poorly in LLC-MK_2 cells also); the third was lost because of a defective culture flask. It is apparent that the mosquito cells are a satisfactory system for rapid detection of at least 3 of 4 dengue serotypes and that their use should be further explored.

Considerable progress in developmental tick tissue culture was made this year, with the apparent establishment in culture of a serially propagating line of cells from D. variabilis embryos. These cells, now in their 21st passage, are diploid and capable of forming confluent monolayers

within one week. At this time, cultures may be split on a 1:2 or 1:3 ratio. This development of a new arthropod ("vector") cell line allows comparison of its virus supporting properties with those of vertebrate ("host") cells. We compared yield of a North American tickborne Flavivirus, Powassan, in the two systems in the presence of a virus inhibitor, glucosamine. This substance, which is a basic component of the glycoprotein of arthropod cuticle, is also known to block the replication of many viruses in vertebrate cells. Duplicate sets of cell cultures of both D. andersoni and Vero, with appropriate controls, were infected with virus. One set was exposed to glucosamine after virus adsorption, while the other did not receive it. Virus increased exponentially in the untreated cultures, but not in those given glucosamine. Cytotoxicity of the amine was not a factor affecting virus growth.

We continued to train investigators from universities and other outside research organizations, both domestic and foreign, and to provide, upon request, arthropod cell cultures, viruses and serological reagents.

Our studies of arthropod-borne pathogens at the cellular level will continue. These will include viral propagation and comparative growth dynamics in vector and host cells, virus attenuation in arthropod cells, and vertical and horizontal transmission of viruses by vectors. Special emphasis will be placed on transovarial transmission, by ticks, of selected viruses. The recent discovery by French workers of natural infections of yellow fever in, and transovarial transmission by, wild caught African ticks has reawakened interest in the tick as a reservoir of mosquito-borne Flaviviruses. Resulting epidemiological implications impinge on the possibility of a tick-St. Louis encephalitis (SLE) virus cycle in the United States. In collaboration with the University of Hawaii, we will investigate transovarial transmission of SLE virus in candidate species of ticks.

Publications:

In press:

Anderson, J. F., Magnarelli, L. A., and Kurz, J.: Hematozoa in rodent populations of Connecticut: Babesia and Grahamella. J. Parasitol.

Bhat, U.K.M. and Yunker, C. E.: Susceptibility of a tick cell line (Dermacentor parumapertus Neumann) to infection with arboviruses. Proc. 2nd International Symposium on Arctic Arboviruses, Mt. Gabriel, Que., Canada, May 26-28, 1977

Yunker, C. E. and Cory, J.: Plaque assay method for arboviruses in mosquito cells. Tissue Culture Manual

Yunker, C. E. and Meibos, H.: Culture of embryonic tick cells (Acari: Ixodidae). Tissue Culture Manual

Appendix 1. PL-480 Project 03-030-N. *In vitro* and *in vivo* Studies of Certain Tickborne Viruses in Vector and Host Cells, Al Azhar University, Cairo, Egypt.

Annual Funding: $97,738

This project seeks to understand the contrasting behavior of a tickborne virus that is pathogenic for humans in Egypt, when grown in a vertebrate host as opposed to the vector. During the second year of the project, eight manuscripts were completed; their subjects include immunosuppression of Quaranfil virus, viremia dynamics in cell culture and comparative behavior of virus in vector and non-vector ticks. Additional lines of study are in progress.

Appendix 2. PL-480 Project 03-051-N. Biochemistry and Physiology of Tick Vectors of Disease.

Annual Funding: $156,294

This project was designed to provide basic biochemical and physiological information on tick metabolism in order to define the environment in which tickborne pathogens of man and animals exist when not infecting their definitive host. Additionally, these data aid in the refinement of tissue culture media specifically designed for tick cells. During the first year of the project, embryonic changes in nucleic acids, proteins, carbohydrates and nitrogenous excretory products were studied. Results revealed at what stage the embryo becomes maternally independent. Manuscripts describing these results are in preparation.

Rocky Mountain Laboratory
Adminstrative and Operational Support
Operations Branch

I979 Annual Report
Table of Contents

October 1, 1978 - September 30, 1979

Administrative and Operational Support for Rocky Mountain Laboratory

Operations Branch

NIAID

Introduction

The Branch provides all services necessary to the professional staff in the pursuit of their investigations. This support covers the following areas; procurement, personnel, custodial, security, media preparations, glassware refinishing, photography, animal rearing and care, motor pool, operation of power plants, and full maintenance in every area except electronics.

General Overview of the Responsibilities of Operations Branch

The fiscal and procurement department manages a budget of $950,000. Payroll is not included in this figure. It covers only the purchase of supplies and minor equipment used in the operation of the laboratories. Timekeeping and the correction of errors in the payroll are also handled in the unit.

Personnel handles all actions and advises on personnel matters. This department also is charged with the operation of the Comprehensive Employment Training Act (CETA) in association with the local Montana State Employment Office. Through the year we have averaged fifteen (15) people on this program at all times. The maximum time a person may spend on the program is 3 months. Hence, we are constantly interviewing and employing people under the program. Also handled by Personnel are persons under the following programs: Stay in School, Work Study, Government Summer Program, and students studying for advanced degrees.

Custodial services are provided in the five laboratory buildings daily. Security is provided in the form of a guard on duty every night of the year.

Most of the media used in the laboratories is prepared by the branch. All glassware used in the lab is cleaned and sterilized for reuse in the laboratories.

The photography department provides full professional services necessary in the laboratories with the exception of medical artistry.

The animal unit raises two strains of guinea pigs, five strains of mice plus one strain of rats and hamsters, and one colony of microtus. They breed and raise approximately 145,000 animals a year. An additional 12,000 animals are purchased annually from outside sources, including mink, sheep, rabbits, mice, guinea pigs, and hamsters. After rearing care is provided for these animals while they are under experiment.

The maintenance department, through the power plant, provides heat, steam, air and vacuum to the laboratories. Also provided is air conditioning, compressed air, vacuum and demineralized and distilled water. A motor pool consisting of 10 vehicles is maintained and grounds care, including snow removal, is provided.

With the exception of the electronics work all maintenance is done by the staff. This includes plumbing, electrical, sheet metal, carpentry, and refrigeration, including ultra low temperature boxes.

Labor management work is handled by the Chief of the Branch.

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