Brugia malayi: IMMUNOBIOLOGY OF MICROFILARIAL CLEARANCE AND THE LESION OF OCCULT FILARIASIS IN FERRETS

By

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Chairman: Richard B. Crandall
Major Department: Immunology and Medical Microbiology

Microfilaremia, peripheral blood leukocyte counts, immune responses and pathology were examined in ferrets injected intravenously with 10^6 *Brugia malayi* microfilariae (mf) or infected with 100-200 third-stage larvae (L3). Clearance of circulating mf was associated with eosinophilia and antibody (IgG or IgM) with specificity for the microfilarial sheath. Passive transfer of sera with IgG sheath-antibody titers $\geq$ 1:200 and microfilarial agglutination titers $\geq$ 1:40 cleared intravenously injected mf or circulating mf established by L3 infection. Sera which cleared *B. malayi* mf did not clear circulating *Dirofilaria immitis* mf. Opsonization of mf in sera containing sheath-reactive IgM antibody resulted in enhanced complement fixation, as compared to mf opsonized in sera containing sheath-IgG antibody, but did not prevent the circulation of mf following intravenous (i.v.) injection. Microfilariae opsonized in sera with sheath-IgG antibody failed to circulate following i.v. injection. Passive transfer of sera

fractions obtained by Sephacryl-300 column chromatography demonstrated that clearance of circulating microfilariae was limited to the IgG-containing fraction of immune sera.

There was a marked similarity of inflammatory responses to mf in ferrets infected with L3 or injected with mf with the inflammation reported in occult infection of man. An association between immediate hypersensitivity to microfilarial antigens and the characteristic lesion of occult infection was observed. In addition, a significant positive linear correlation between the number of surface liver lesions typical of occult filariasis and the extent of blood eosinophilia 1 and 3 weeks following i.v. injection of mf was noted. Ultrastructural and histochemical studies indicated that degranulating eosinophils were major contributors to the characteristic pathology. Passive transfer of sera with microfilarial-specific reaginic antibody induced a significant increase in the number of ferrets which exhibited typical lesions 1 week following i.v. injection of mf. However, lesions of occult filariasis developed in the absence of detectable immediate hypersensitivity to microfilarial antigens in ferrets infected 30 days with Trichinella spiralis and then injected with mf. These results indicate that although immediate hypersensitivity to microfilarial antigens probably represents an immune mechanism active in the pathogenesis of occult infection, the typical lesion may be induced by other immunologic mechanisms.

INTRODUCTION

Tropical diseases, especially the parasitic diseases, in the developing countries of Africa, Asia and South America kill or disable millions of individuals; more people suffer and die from tropical parasitic diseases than all other diseases combined. The five most severe tropical parasitic diseases are malaria, trypanosomiasis, leishmaniasis, schistosomiasis and filariasis (1). Malaria, schistosomiasis and filariasis afflict 150, 200 and 300 million people, respectively and malaria each year kills more than 1 million children in Africa (2). Tropical parasitic diseases also infect domestic animals which often result in serious economic loss, increased poverty and malnutrition. Clearly, tropical parasitic diseases can create a vicious cycle leading to major health problems and an obvious need exists for effective control measures to prevent disease transmission and knowledge to prevent the severe pathology associated with these diseases.

One of the most debilitating tropical parasitic diseases is filariasis. Filariasis is an infectious disease of man and animal caused by nematodes of the Order Filariidae (3). The human filarial parasites are commonly classified into three groups based on the tissue location of the adult parasites: the cutaneous group (Loa loa, Onchocerca volvulus, and Dipetalonema streptocerca), the body cavity group (Dipetalonema perstans and Mansonella ozzardi), and the lymphatic dwelling filariae (Wuchereria bancrofti, Brugia malayi, and

Brugia timori). Loa loa, Onchocerca volvulus and the lymphatic dwelling filariae are responsible for most of the filarial induced pathology observed in man, but knowledge of the pathogenesis of these diseases is minimal (4).

The pathology induced by the lymphatic-dwelling filariae can be localized or systemic. Localized pathology is confined to the lymphatic vessels containing adult parasites and the associated draining lymph nodes; this pathology, in its most severe form, can result in chronic lymphatic obstruction and elephantiasis. Systemic pathology is the result of immflammatory responses to microfilariae trapped within the lung, liver and spleen. The role of the immune system in the pathogenesis of chronic lymphatic obstruction and the systemic inflammatory responses to trapped microfilariae has not been well defined. To identify pathophysiologic mechanisms active in filarial disease, controlled conditions and appropriate experimental animal models are essential. The inflammatory cell response of the ferret to Brugia malayi microfilariae is morphologically identical to the characteristic histopathology observed in individuals expressing clinical signs of systemic filarial disease (tropical pulmonary eosinophilia or the Meyers-Kouwenaar syndrome) and is unique among all experimental animal models examined (5). Using the ferret as an experimental animal model, the objective of this research was to identify the immune mechanism of microfilarial clearance and to elucidate the immunopathogenesis of the inflammatory response to trapped microfilariae.

BACKGROUND REVIEW

Lymphatic Filariasis

The lymphatic dwelling filariae (*W. bancrofti*, *B. malayi* and *B. timori*) are medical and public health problems in many tropical and subtropical areas. Clinical manifestations of infection vary from the asymptomatic to the most familiar presentation, elephantiasis. Although investigations indicate that immune responses play an important role in susceptibility, pathogenesis and acquired resistance in filariasis, knowledge of the immunologic mechanisms remains fragmentary (4,6,7).

Vectors and Geographic Distribution

Known vectors of the lymphatic dwelling filariae are mosquitoes of four genera: *Anopheles*, *Aedes*, *Culex* and *Mansonia* (3). Transmission of disease is most common in tropical and subtropical regions because the vectors fail to thrive in other areas. *W. bancrofti* has the widest geographical distribution and transmission has been recorded throughout much of the tropics and subtropics with the exception of very dry areas or desert regions (3); in the Western Hemisphere, an endemic focus of *W. bancrofti* was established as far north as Charleston, S.C., but due to increased sanitation and an intensive mosquito control program, the disease was eliminated more than 50 years ago (8-10). *B. malayi* is endemic exclusively in South

and East Asia and B. timori is known to be endemic only in Timori and the Flores Islands of the Indonesian Archipelago (3).

Life Cycle

Wuchereria and Brugia filarial parasites have five developmental stages and completion of the life cycle requires 3 to 6 months (Fig. 1). The fifth-stage, or adult worms, reside in the lumen of lymphatic vessels where they may live for years (11,12). B. malayi adult worms in man were first described by Rao and Maplestone in 1940 (13). The mature females vary from 43.5-55.0 mm in length and 0.13-0.17 mm in width, and males from 13.5-23.3 mm in length and 0.07-0.08 mm in width (14). The adults of W. bancrofti, initilly described by Cobbold in 1877 (15), have a similar thread-like appearance, but are approximently twice the size of B. malayi in both length and width (16). After mating, the adult female releases microfilariae, or first-stage larvae, into the lymphatic lumen; these microfilariae are approximently 0.2-0.3 mm long depending on the species. The microfilariae make their way through the lymphatic system to the peripheral blood where they circulate in a periodic or subperiodic manner depending on the species or race of parasite. Microfilarial periodicity was recognized by Manson in 1879 when he observed that patients with a nocturnal microfilaremia failed to demonstrate microfilariae in the blood during the day (17); periodic microfilaremia is defined as the appearance of microfilariae in the peripheral blood only during certain periods of the day and subperiodic microfilaremia as microfilariae present in the blood throughout the day with peak microfilaremia occurring at specific

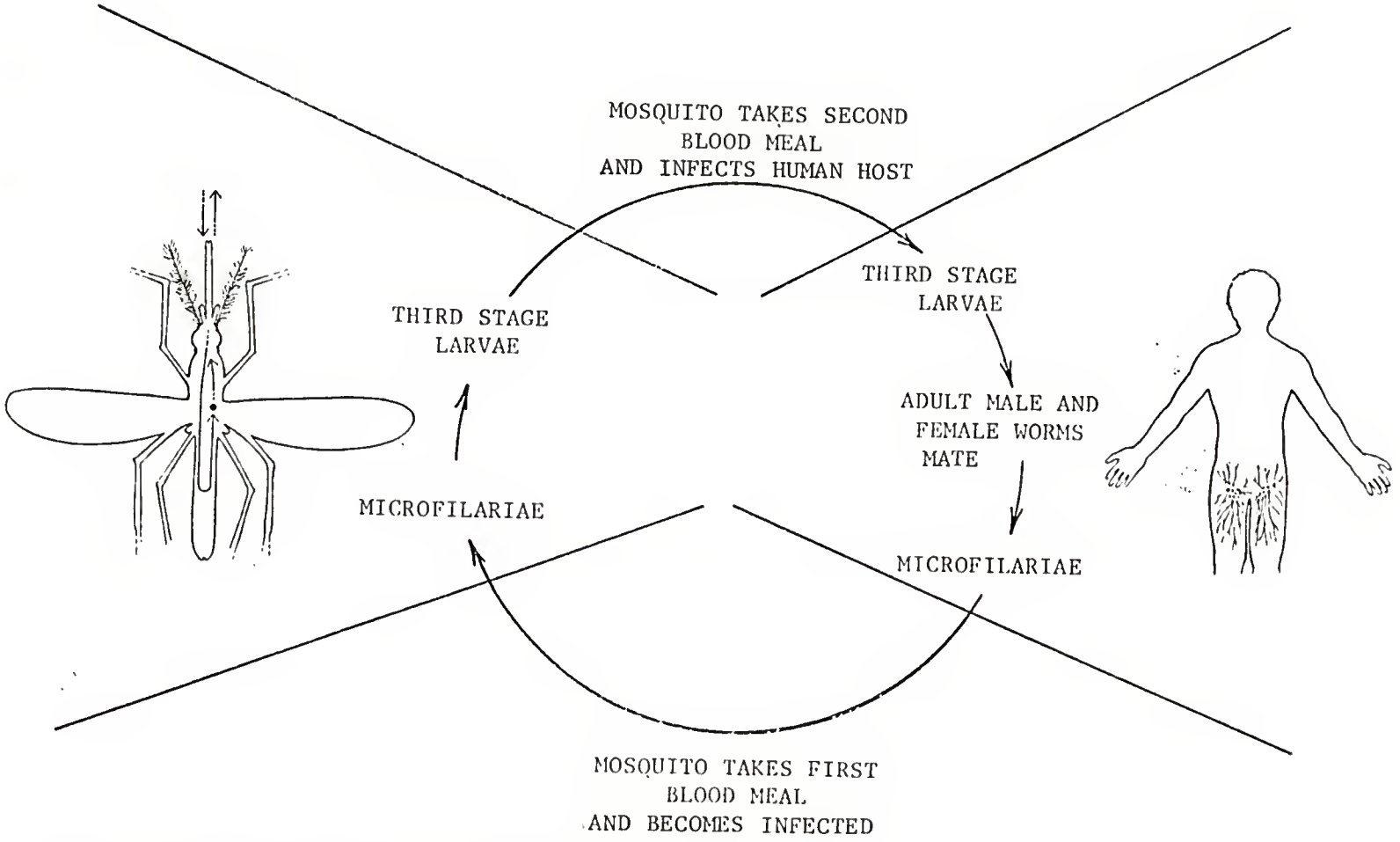


Fig. 1. LIFE CYCLE OF LYMPHATIC DWELLING FILARIAE.

times. Most investigators suggest that periodicity is a result of microfilarial retention in the pulmonary capillaries and their subsequent release into the peripheral blood during certain hours of the day (18). Microfilariae of W. bancrofti have been recorded as nocturnally periodic, nocturnally subperiodic, and diurnally subperiodic (19), while B. malayi have been recorded as only nocturnally periodic or subperiodic (20,21). The mosquito vectors of these parasites also have a circadian rhythm in which they feed at specific periods of the day. The highest concentration of microfilarae in the peripheral blood occurs when the local mosquito vector is most actively feeding (22). The mosquito is an obligate intermediate host for lymphatic dwelling filariae and microfilariae must be ingested before they can molt to the next larval stage; transmission of filariasis by mosquitoes was the first demonstration that insects may serve as intermediate hosts for mammalian infections (17). Following ingestion, the microfilariae migrate through the gut wall and enter the thoracic muscles. The microfilariae then molt twice to the infective, or third-stage, larvae over the period of 1-2 weeks and then force their way out of the thoracic muscles and migrate to the proboscis. Disease transmission occurs when the mosquito takes a subsequent blood meal. The infective larvae migrate onto the surface of the skin, enter the host through the wound created by the mosquito proboscis, and migrate into the lymphatics where they molt twice to become adolescent and finally, reproducing adults.

Clinical Manifestations of Infection

The clinical manifestations of lymphatic dwelling filarial infection are variable among individuals residing in endemic areas [Fig. 2 (copied from Ottesen, 4)]. Epidemiological studies of the number of mosqutio bites received by individuals and entomological investigations of the number of mosquitoes which contained infective larvae have indicated that nearly all individuals in rural endemic areas have been exposed to filariasis (22-25), yet a significant proportion of these adults never exhibit clinical or parasitological evidence of infection (26-28); these individuals represent one extreme in the broad spectrum of clinical presentations. Field researchers, collecting immunologic data from endemic populations, have classified these individuals as "normals" or as "exposed but not infected" (4); these data must be cautiously interpreted, because current diagnostic techniques may not detect all subclinical infections.

A second asymptomatic manifestation of lymphatic dwelling filarial infection is characterized by the presence of microfilariae in the peripheral blood. Individuals with this clinical presentation have been defined as asymptomatic microfilaremics and they represent a considerable proportion of residents in endemic areas. How long these individuals remain asymptomatic and microfilaremic and what predisposes them to develop clinical symptoms of filarial disease are intriquing questions whose answers are unknown.

The most common clinical manifestation of symptomatic filariasis is periodic fevers accompanied by lymphadenitis and lymphangitis and these symptoms have been recorded from individuals with or without

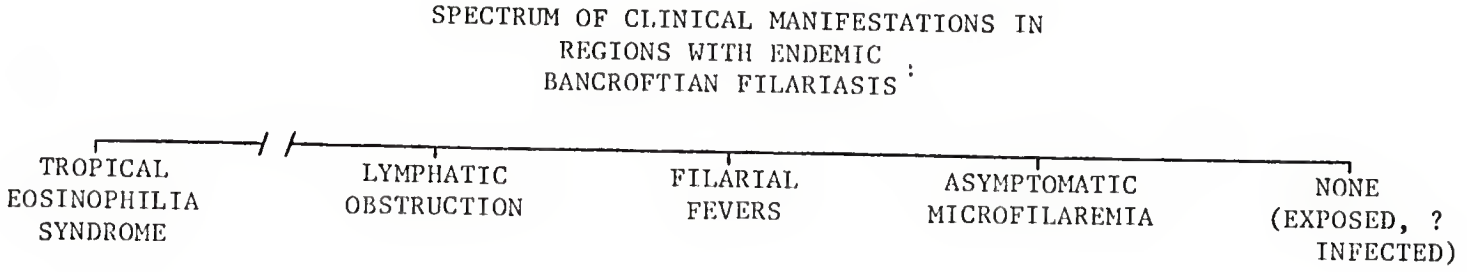


Fig. 2. CLINICAL SPECTRUM OF THE LYMPHATIC FILARIASES (as copied from Ottesen, 4).

circulating microfilariae. These episodes of "filarial fever" last about a week and occur 2-6 times each year. Factors which initiate these acute episodes and which are responsible for the spontaneous remission of symptoms have not been defined. Each episode of filarial fever is followed by post-inflammatory scarring of the associated lymphatic vessel. These episodes of lymphatic scarring have been suggested as the probable cause of the most familiar of the clinical diseases caused by infection with W. bancrofti and B. malayi: chronic lymphatic obstruction with hydrocoel, chyluria and/or elephantiasis. Individuals with chronic lymphatic obstruction rarely demonstrate circulating microfilariae and it is tempting to suggest that the pathogenesis of lymphatic obstruction may involve a microfilarial-specific immune response.

A final clinical manifestation of lymphatic filariasis has been variously termed by physicians and researchers alike as tropical (filarial) eosinophilia, eosinophilic lung, tropical pulmonary eosinophilia, occult filariasis, filariasis without microfilaremia, or the Meyers-Kouwenaar syndrome. This disease is characterized by extreme blood eosinophilia associated with a paroxysmal cough, scanty production of sputum and an interstitial pulmonary cell infiltrate on radiographic study. Microfilaremia is essentially never seen in this clinical disease presentation. However, microfilariae are produced by the adult worms and can be observed in various tissues, including pulmonary tissue; the histopathologic response to entrapped microfilariae is characteristic and will be described later.

Parasite-Induced Immune Responses

Knowledge of the immune responses in man to filarial infection has been obtained through skin tests, antibody assays, and *in vitro* methods of assessing lymphocyte responses. Data collected using these techniques indicate that immune responses to parasite antigens can vary extensively among individuals with lymphatic filariasis. However, many studies have used parasite antigens extracted from non-human filariae and the biological significance of these responses is not known. Studies of immune responses to homologous antigens are limited and have not been well characterized. Immunologic responses to most pathogens fall into one of three categories (protective, pathologic or suppressive immune responses) and study of host immune responses to filarial parasites has followed this trend.

Protective immune responses

Infection with lymphatic dwelling filarial parasites expose the vertebrate host to several stages of the parasite life cycle. Protective immune responses could be aimed at any of these stages, but immunity to the initial infective stage would have obvious consequences in the prevention of parasite development.

Immunity to infective larvae. Studies of immune responses in man to third-stage larvae have been limited, due mainly to difficulty in obtaining this stage from infected mosquitoes, and have centered on *in vitro* studies of humoral substances which promote cellular adherence to the larvae (29-32). These studies have demonstrated the presence of humoral substances, in sera collected from patients with tropical pulmonary eosinophilia or elephantiasis, amicrofilaremic

individuals with a history of filarial fevers, and normal individuals living in endemic areas, capable of initiating *in vitro* cell-mediated damage to infective larvae. *In vivo* resistance to infective larvae has been studied using a *B. pahangi* cat model of filariasis. Cats repeatedly infected with *B. pahangi* were examined for resistance to infective larvae 1 day following a secondary infection; uninfected control animals demonstrated 50-60% recovery of challenge larvae, while 1% recovery was considered high in the previously infected animals (22).

Immunity to microfilariae. Antimicrofilarial immunity was first reported by Pandit and coworkers in 1929 (33). They described the presence of a humoral factor in sera from most patients with amicrofilaremic filariasis which promoted leukocyte adherence to microfilariae. Since that time several investigators have described striking correlations between the presence of microfilarial sheath reactive antibodies in serum and the absence of circulating microfilariae (34-36); anti-sheath IgG, IgM, and IgA antibodies have been reported (37). Further studies have identified a dissociation between cellular and humoral immune responses to microfilarial antigens in single individuals (34,38); i.e., amicrofilaremic individuals demonstrate anti-sheath antibodies or a marked *in vitro* lymphocyte blastogenic response to microfilarial antigens, but not both. Investigations utilizing animal models of filarial infections have also demonstrated that antibodies to the surface of microfilariae are associated with amicrofilaremia and that these antibodies may be involved in the regulation of microfilaremia (39-48).

The eosinophil in protective immunity. Eosinophils have been observed in hypersensitivity reactions including anaphylaxis (49) and Arthus reactions (50), and have long been associated with helminth antigens: first recorded in 1891 from studies of patients harboring hookworm infections (51). Eosinophilia is most often observed during the invasive or migratory stages of the parasite life cycle and may become chronic if a persistent tissue inflammatory response occurs (52). The role of the eosinophil in resistance to parasitic helminth infection has primarily been accomplished in detailed studies of mice and rats infected with Schistosoma mansoni (53-56). Histologic studies implicate the eosinophil in resistance to challenge infection as an eosinophil-enriched inflammatory response around the schistosomula has been demonstrated in the skin of experimental animal models (57-60); although direct in vivo contact has not been observed. Resistance involves an IgG fraction of immune serum and a radiosensitive cell (60-62). Resistance may be blocked by prior treatment of experimental animals with an anti-eosinophil serum (63) which implicates the eosinophil as the potential killer cell in the antibody-dependent cell-mediated immune response. Histologic studies in man have demonstrated the close proximity of degranulating eosinophils to microfilariae of Onchocerca volvulus in the skin of patients treated with diethylcarbamazine (64). The ability of the eosinophil to degranulate into the extracellular medium when confronted with large antigen-antibody complexes (65) suggests that eosinophil exocytosis may be a host immune response against nonphagocytosable pathogens.

Immune responses and the pathology of filariasis

The pathology associated with lymphatic dwelling filarial parasites apparently is induced by the clearance of circulating microfilariae in hypersensitive individuals and the inflammatory responses to maturation and/or death of adult worms within the lymphatic vessels.

Immunopathology associated with microfilarial clearance. Circulating levels of microfilariae in individuals with asymptomatic microfilaremic infections are relatively constant for many months as indicated by epidemiological investigations. Apparently these individuals are able to continually clear circulating microfilariae at a rate equal to their release from gravid females to keep their microfilarial burdens constant, and in these instances, microfilarial clearance is not associated with clinical manifestations of disease. In addition, infected individuals who develop sheath-reactive antibody can become amicrofilaremic without evidence of clinical disease. However, clearance of microfilariae can be associated with severe tissue reactions and this type of occult filariasis is characterized by immunologic hyperresponsiveness to microfilarial antigens, marked eosinophilia, and high concentrations of IgE (4). Occult filariasis can be associated with asthmatic symptoms (tropical pulmonary eosinophilia) and/or with lymphadenopathy and occasionally hepatosplenomegaly (Meyers-Kouwenaar syndrome). The histopathologic response to entrapped microfilariae is characteristic and has been found in lymph nodes, lungs, liver and spleen. These lesions are small whitish foci, with diameters of 1-5 mm. Microscopic examination demonstrates aggregates of eosinophils and in the center of these

aggregates, microfilariae or remnants of microfilariae may be found. Acidophilic hyaline material, termed Splendore-Hoeppli substance, often surrounding dead microfilariae may also be seen in the center of eosinophil collections. The microfilariae and surrounding Splendore-Hoeppli substance are known as Meyers-Kouwenaar bodies (66,67). The high degree of allergic hypersensitivity to antigens of microfilariae is considered to be a probable cause of this disease, but experimental studies of immunopathogenesis are lacking. It is unclear what predisposes certain individuals to react to entrapped microfilariae in a hyperresponsive manner in contrast to the individual who lacks clinical symptoms of disease following the clearance of microfilariae.

Immunopathogenesis of lymphatic obstruction. Specific immune mechanisms responsible for the lymphatic pathology observed in filariasis are largely undetermined. Investigation has been difficult because the acute episodes of lymphangitis and lymphadenitis in man occur, at best, 2-6 times yearly; however, valuable information has been gained from cats and dogs experimentally infected with Brugia species. These animal models demonstrated radiographic and direct anatomic evidence of lymphatic pathology primarily when the developing larvae were molting and subsequently releasing highly antigenic material (68); however, the immunologic mechanism and the precise antigens involved in the pathogenesis have not been defined. Observations in man have suggested that cell-mediated immune responses may be involved in filarial lymphatic pathology. Piessens et al. (38) identified marked in vitro lymphocyte blastogenic responses to adult filarial antigens in patients with lymphatic pathology, but only

minimal sensitivity in other infected individuals not exhibiting lymphatic pathology. Other evidence for the participation of cellular immune mechanisms in lymphatic pathology has stemmed from research in jirds infected with B. pahangi (4); chronic injection of antithymocyte serum into infected jirds resulted in decreased lymphatic pathology. These experiments implicate cellular immune mechanisms in the pathology of lymphatic filariasis, but clearly additional study is required.

Parasite-induced immunosuppression

A critical factor in the transmission of lymphatic filariasis is the presence of microfilariae in the peripheral blood. Unabated microfilaremia may be the result of specific stimulation of the host regulatory immune system leading to suppression of immunologic mechanisms involved in microfilarial clearance. Specific immune unresponsiveness to microfilarial antigens has been recorded in human infections with W. bancrofti and B. malayi and was most evident in patients with microfilaremia (38,69). Piessens et al. (70,71) have demonstrated three mechanisms that may regulate the host immune response to parasite antigens during infection with B. malayi: (1) adherent suppressor cells, (2) nonaderent suppressor cells and (3) serum factors. These experimental studies demonstrated an increase in the proportion of peripheral blood T-lymphocytes of the suppressor phenotype in patients with microfilaremia due to Brugian filariasis. This increase in total numbers of suppressor T-lymphocytes was due mainly to an increase in populations of cells that specifically inhibited immune reactions to filarial antigens. These investigators

have also identified filarial specific suppressor cells which were phagocytic and adherent as well as suppressor factors in sera from microfilaremic patients (70). The suppressor factor in serum has not been defined and, unlike the antigen-specific suppressor cells, may exert a nonspecific suppression of the immune response. Several humoral substances which are elevated during acute and chronic inflammatory diseases have been reported to nonspecifically suppress immune reactions (72). Unfortunately, the role of filarial induced immune suppression in the chronicity of filarial infection remains unanswered.

Statement of the Problem

To identify immune responses active in the pathogenesis of filarial disease or resistance to infection, long term studies of controlled experimental infections are essential; however, adequate experimental models for the lymphatic dwelling filariae of man are limited and none exhibit the diverse manifestations of human disease. Both *Brugia malayi* and *Brugia pahangi* have been used in laboratory animals as experimental models (22). Inbred strains of rodents are of obvious value for immunologic investigation and important information has been obtained with their use, but the host-parasite relationship in these animals differs from natural hosts (i.e., adult parasites are not confined to lymphatic vessels) and direct application of experimental results to man may be questioned. Certain carnivores, particularly cats, are natural hosts for *Brugia* and have been useful experimental animals but there is still a need for an economical,

small experimental host in which the history of infection parallels that of natural hosts, and which the manifestations of human disease may be duplicated.

The ferret is a small carnivore which is commercially available, relatively inexpensive, and easily handled in the laboratory. The ferret has been used as an experimental host to study filarial infections: Dirofilaria immitis, Brugia pahangi and Brugia malayi (5,73,74). Preliminary studies suggested that a significant characteristic of Brugia infection in the ferret was the combination of amicrofilaremia, persistent eosinophilia, and inflammatory cell response to microfilariae which were similar to the characteristic findings in tropical (filarial) eosinophilia or the Meyers-Kouwenaar syndrome caused by occult filariasis in man (5). These characteristics indicated that the ferret could be a potentially useful experimental animal model for the investigation of immunologic mechanisms active in occult filariasis.

This research was designed to study the immunobiology and pathology of B. malayi infection in the ferret. The investigation examined the immune responses, total and differential leukocyte counts, microfilaremia and pathology in ferrets infected with B. malayi third-stage larvae and/or injected intravenously with microfilariae. The intravenous injection of microfilariae was used to facilitate experimental study by eliminating the time required for maturation of infection, confine host stimulation to microfilariae, and permit experimental control of the infection by varying both the timing and amount of microfilariae injected. The specific aims of the research were (1) to evaluate the ferret as an experimental animal

model for the study of tropical (filarial) eosinophilia or occult filariasis in man and (2) to identify the immune mechanisms of microfilarial clearance and the immunopathogenesis of the lesion associated with occult filariasis in the ferret.

MATERIALS AND METHODS

General Procedures

Animals and Infections

Jirds were purchased from Tumblebrook Farms, Inc., West Brookfield, MA, and were injected with L3 either subcutaneously or intraperitoneally by J. W. McCall (University of Georgia, Athens, GA). Jirds injected subcutaneously with L3 were used as a source of peripheral blood microfilariae to infect mosquitoes and those jirds injected intraperitoneally with L3 were used for the collection of microfilariae by peritoneal lavage.

Intact male ferrets (6 months - 1 year of age) were purchased from Marshall Research Animals, Inc., North Rose, NY. Intravenous (i.v.) injection of microfilariae (mf) into ferrets was carried out by catheterization of the external jugular vein. Ferrets were sedated with an intramuscular injection of ketamine hydrochloride (Veterinary Products, Bristol Laboratories, Syracuse, NY) at 50 mg/Kg body weight. The neck was prepared for sterile catheterization, and a 22 gauge, 8 inch Intracath catheter (Deseret Medical, Inc., Park, Davis and Co., Sandy, UT) was inserted percutaneously. A 30 ml solution of sterile phosphate buffered physiological saline (PBS) containing mf was then administered at a rate of 0.5-1.0 ml per minute. Infection of ferrets with third-stage *B. malayi* larvae was accomplished by subcutaneous injection of 50 L3 in the dorsum of both rear paws; numbers of larvae inoculated were determined by counting the larvae added to the syringe and the larvae remaining following inoculation.

Recovery of Larval Stages

Aedes aegypti were raised in an insectory in the Health Center, and infected with Brugia malayi mf by feeding on infected jirds or with a membrane feeding apparatus (McCall, personal communication). Recovery and handling of L3 from mosquitoes followed described procedures (75). Microfilariae were obtained from the peritoneal cavity of infected jirds by peritoneal lavage (24). The lavage fluid was passed through a lightly packed column of nylon wool to remove cell clumps and dead mf. The suspension was then passed through an 8 um Millipore filter (Millipore Corp., Bedford, MA); jird peritoneal cells pass through the filter, but not mf. The mf were then washed off the filter using a stream of PBS. The resulting microfilarial suspension contained approximately 11 mf/jird peritoneal cell. These mf were used for i.v. injection of ferrets, indirect fluorescent antibody tests and for antigen preparation.

Microfilariae of D. immitis were collected from a single dog. A peripheral blood sample was allowed to clot and the sera, containing D. immitis mf, was then passed through an 8 um Millipore filter. The mf were then washed off the filter using a stream of PBS as described above.

Preparation of Microfilarial Antigen

Washed samples of B. malayi microfilariae were suspended in 0.1 M Tris buffer pH 8.0 at 1 volume worm material to 5 volumes buffer. The parasite suspension was homogenized by several passages through a French pressure cell at 20,000 psi. The homogenate was extracted overnight with gentle shaking and the aqueous soluble component

isolated by centrifugation for 30 minutes at 40,000 g. The supernatant was concentrated to 1 mg/ml of protein (Bio Rad protein assay, Bio Rad Laboratories, Richmond, CA) by ultrafiltration on a YM-10 filter (Amicon Corp., Lexington MA). All manipulations were done at 4°C and the supernatant stored at -20°C.

Microfilaremia and Leukocyte Counts

Ferrets were routinely bled between 1000-1400 hours from the jugular vein using a 21 gauge vacutainer needle and tube. Microfilaremia was determined by direct microscopic examination of 50 ul blood and total leukocytes determined by diluting the blood in a Unopette (Becton-Dickinson, Rutherford, NJ) and manually counting the leukocytes on a hemocytometer (American Optical, Buffalo, NY). Differential leukocyte counts were determined on cover-slip blood smears stained with Diff Quik (American Scientific Products, McGaw Park, IL). Serum samples were obtained at each bleeding and stored at -20°C.

Exploratory Laparotomy and Wedge Liver Biopsy

Ferrets were sedated with 50 mg/Kg ketamine hydrochoride intramuscularly, and pentobarbital sodium was then administered intravenously at 5-7 mg/Kg body weight. The abdomen was prepared for aseptic surgery using alternate betadine and alcohol scrubs, was toweled off and then draped. A number 15 scalpel blade was used for skin incision and a combination of blunt and sharp dissection was used to expose the linea alba. The midline was tented and a stab incision made. The incision was carried rostrally and caudally with sharp

dissection. The abdomen was examined following standard procedures (76). A pair of horizontal mattress sutures were preplaced in the liver using 5-0 silk on an atraumatic needle; wedge liver biopsy was performed between these sutures and the cut liver edges immediately closed. The abdomen was reexamined for bleeding and was closed in three layers using standard technique (76).

Histology

Tissues collected at necropsy and exploratory laparotomy were processed for light and/or electron microscopy. Tissues for routine light microscopy were cut approximately 75-100 cu mm and were fixed in 10% buffered formalin, embedded in paraffin and sectioned following described procedures (77). Sections were routinely stained with hematoxylin and eosin; selected sections were also stained with Giemsa, Gomori methanamine-silver, Movat or Brown-Hopps (78).

Tissues examined for endogenous peroxidase were frozen and sections cut on a cryostat (International Equipment Co., Needham Hts., MA). Frozen sections were fixed in 10% formalin in 95% ethanol for 2 minutes, then immersed for 10 minutes in a 0.05% solution of 3,3' diaminobenzidine hydrochloride (Sigma Chemical Co., St. Louis, MO) in PBS containing 0.01% hydrogen peroxide. Sections were counterstained with Gill's hematoxylin (Fisher Scientific Co., Pittsburgh, PA) after washing in running tap water.

Tissues for electron microscopic study were cut approximently 5-8 cu mm and were fixed in formaldehyde-glutaraldehyde according to McDowell and Trump (79). Post-fixation processing was performed as described by Dykstra and Hackett (80). Tissues were embedded in

Spurr's plastic (81). Ultrathin sections were cut on a Sorvall MP 5000 microtome and stained with methanolic uranyl acetate followed by lead citrate (82). Stained sections were examined on a Zeiss DM 10 electron microscope.

Immunologic Procedures

Antisera

Antisera to ferret IgM and IgG were prepared in rabbits following described procedures (83). Briefly, ferret serum was filtered through a Protein A-coupled Sepharose 4B (Pharmacia Fine Chemicals, Pharmacia Inc., Piscataway NJ) column; bound immunoglobulin was then eluted and gel-filtered on Sephacryl-300 (see Chromatography below). Protein fractions were concentrated by ultrafiltration on a YM-10 filter (Amicon Corp., Lexington, MA) and analyzed for purity by immunoelectrophoresis. Rabbits were injected subcutaneously in multiple sites with IgG or IgM emulsified in Freund's complete adjuvant (84). Rabbit antiserum to ferret IgM was adsorbed with ferret IgG; following adsorption, this antiserum gave a single IgM precipitin band on immunoelectrophoresis with ferret serum. Rabbit antiserum to ferret IgG showed a major IgG precipitin band and only minor cross-reactivity with other immunoglobulins.

Chromatography

Ferret sera and immunoglobulins previously eluted from a Protein A-coupled Sepharose column were gel-filtered on a Pharmacia column containing an 80 x 2.5 cm bed of Sephacryl-300 (Pharmacia Fine Chemicals, Pharmacia Inc., Piscataway, NJ); sera and immunoglobulins were passed through 0.22 um Millipore filters (Millipore Corp., Bedford, MA) prior to chromatography. Column chromatography was carried out at room temperature with 0.05 M Tris buffer pH 7.8 and 0.15 M NaCl, at a rate of 12 ml/hr; 0.02% sodium azide was added to the buffer to prevent contamination. Eluates (2.5 ml) were collected with a Gibson micro-fractionater.

Immunofluoresence

The indirect flourescent antibody technique (IFAT) was used to detect antibody to microfilarial sheaths following the techniques of Wong and Guest (36). Antisera to ferret Ig classes were labeled with fluorescein isothiocyanate as previously described (84).

Microfilarial Agglutination

Microfilarial agglutination was performed using 3000 B. malayi mf and dilutions of test sera in PBS at a final reaction volume of 0.2 ml as an alternative method to the IFAT for the detection of surface-reactive antibody. Incubation was carried out at room temperature for 30 minutes with occasional shaking. Agglutination was determined by microscopic examination.

Hypersensitivity to Microfilarial Antigens

The Prausnitz-Kustner (PK) test was used to identify sera containing IgE antibody to microfilarial antigens. The procedure followed that described for testing IgE antibody to S. mansoni antigens (85). One milliliter of 1% Evan's blue dye in PBS was injected intravenously and then 0.2 ug protein (Bio-Rad protein Assay, Bio Rad Laboratories, Richmond, CA) of a saline extract of Brugia malayi mf was injected intradermally. The area of the skin test response, jugded by the extent of visable blue dye, was measured 15 minutes following intradermal injection of antigen. Direct skin test for hypersensitivities was done similarly to the PK test except 2.0 ug, 0.2 ug, and 0.02 ug microfilarial protein was used to identify the degree of hypersensitivity; skin test results were read at 15 minutes and 24, 48, and 72 hours following intradermal injection of antigen.

Complement Fixation

Normal ferret blood was collected by venipuncture, was placed on ice and the serum separated immediately after clot formation. Aliquots of serum were then frozen at -70° C. Titration of the complement activity in normal serum was performed according to described procedures (86).

Complement fixation assays were carried out with live B. malayi microfilariae or microfilariae which were fixed overnight in 2% buffered formalin at 4° C and washed in PBS the following day. Dilutions of immune or normal ferret sera were incubated with 105 mf for 1 hour at room temperature with occasional shaking in a final volume of 2 ml PBS. Microfilariae were then washed twice with 15 ml

PBS and once with 15 ml veronal buffered saline containing 0.1% gelatin and 0.001 M Mg++ and 0.00015 M Ca++ (VBS). Microfilariae were resuspended into 1.0 ml VBS. Two-fold serial dilutions of 0.4 ml microfilarial suspension were performed from 1:1 to 1:128 and then 2.2 ml of a 1:660 dilution of normal ferret serum was added to each tube. Tubes were incubated at 37°C for 1 hour with occasional shaking. Following incubation, 0.4 ml of $1.5X10^8$ sheep red blood cells per ml VBS were added to each tube; sheep red blood cells were opsonized with murine monoclonal IgM anti-Forrsman antibody, kindly supplied by Dr. Michael Boyle. Tubes were then incubated at 37°C for 30 minutes. At the end of 30 minutes, tubes were centrifuged and the amount of free hemoglobin in the supernatant determined spectrophotometrically at a wavelength of 405 nm.

Western Blot

Antibody responses to isolated microfilarial antigens were studied by electrophoretic transfer of electrophoresed parasite proteins from sodium dodecyl sulfate (SDS) polyacrylamide gels to unmodified nitrocellulose following recommended procedures (87). Briefly, microfilarial antigens were reduced and alkylated at 65°C for 15 minutes in 2.5% SDS, 2.25 M urea, and 0.005 M dithioerythritol. Microfilarial antigens were then electrophoresed at 18°C in a 12.5% polyacrylamide gel using 4 mA constant current. Following electrophoresis, antigens were transferred to nitrocellulose paper using a Trans-Blot cell (Bio Rad Laboratories, Rockville Centre, NY) at 4°C for 10 hours under constant voltage, 60 volts. The nitrocellulose paper was removed, cut into appropriate lanes, and

washed in PBS-Tween. Nitrocellulose strips were exposed to a 1:50 dilution of ferret sera for 1 hour, washed, and then reacted for 1 hour with a 1:100 dilution of Protein A-coupled horseradish peroxidase (Cappel Laboratories, P.O. Box 1867, West Chester, PA). Following incubation, the nitrocellulose strips were washed and then exposed to the substrate and developed according to described procedures (87).

RESULTS

Infection with Third-Stage Larvae

Initial study assessed the course of microfilaremia, total and differential leukocyte counts, and antibody responses to microfilariae in ferrets infected with B. malayi third-stage larvae.

Primary and Secondary Infection

Following a subcutaneous inoculation of 100 third-stage larvae, 14 of 16 ferrets became microfilaremic during the third month post infection; 1 ferret became microfilaremic during the fourth month and 1 never became patent. The maximum height of microfilaremia ranged from 0-6260 mf/ml with a mean of 1068 mf/ml. Persistence of microfilaremia was determined in those ferrets which became patent. Four of 15 ferrets remained microfilaremic at 1 year and 11 ferrets cleared mf by 190 $\pm$ 25 days with a mean patency of 123 $\pm$ 25 days. An absolute eosinophilia developed at patency, and peak eosinophil counts ranged from 1092-5700 eosinophils per microliter blood. Figure 3 shows the microfilaremia and eosinophil counts in 6 ferrets which are representative of the variations observed in the 15 ferrets described above. Sheath-reactive antibody was not detected during microfilaremia. Following microfilarial clearance, 9 of 12 ferrets had detectable levels of either IgG (7 ferrets) or IgM (2 ferrets) sheath-reactive antibody, but not both. In the remaining amicrofilaremic ferrets, sheath-reactive antibody was not detected.

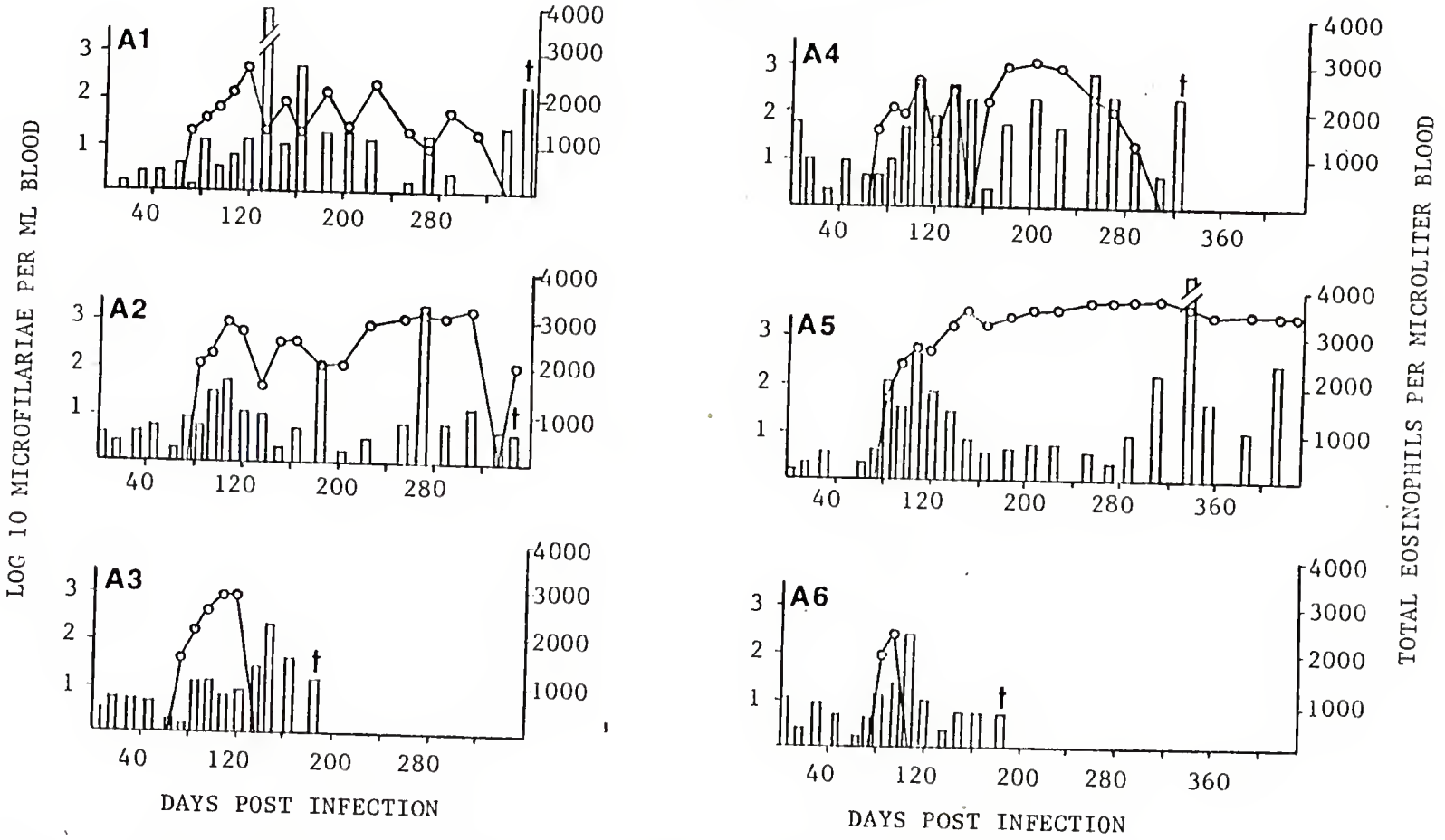


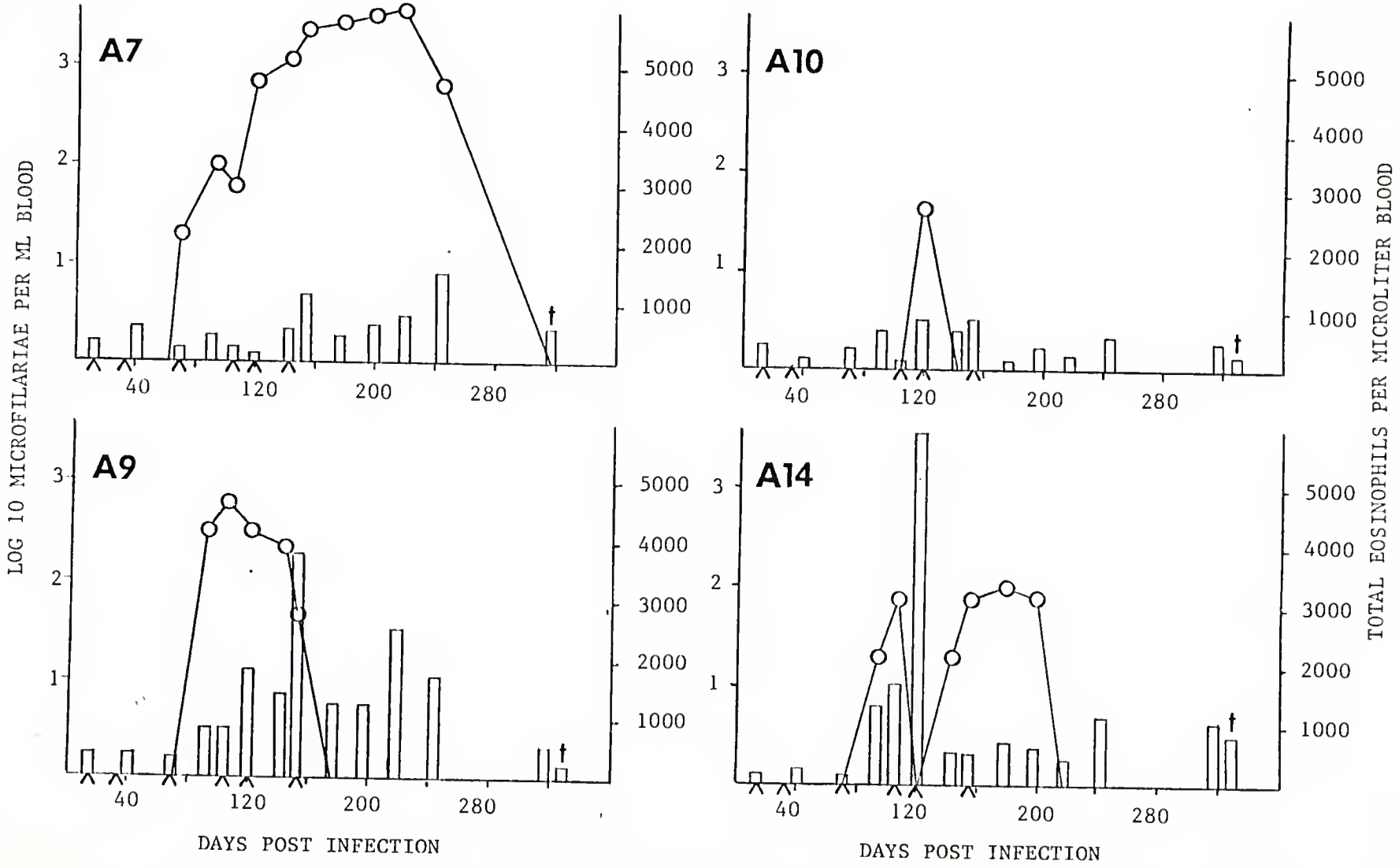
Fig. 3. MICROFILAREMIA AND EOSINOPHILIA FOLLOWING INFECTION WITH 100 B. MALAYI THIRD-STAGE LARVAE. Open bars represent total eosinophil counts and open dots represent numbers of circulating microfilariae. Cross (t) indicates termination of the experiment.

Three ferrets which had cleared circulating microfilariae (mf) were challenged with an infection of 200 B. malayi third-stage larvae; a single control ferret received a primary infection. Peripheral blood was collected every 2-3 weeks for 24 weeks. Microfilaremia was not detected in previously infected ferrets; however, circulating mf were observed in the control ferret. Eosinophilia in the control and previously infected ferrets was transient and occurred 50-115 days following challenge infection. The maximum level of eosinophilia varied from 918-1791 eosinophils per microliter blood. Sheath-reactive IgG antibody was detected in each ferret at 170-172 days following challenge infection; sera were not examined for IgM sheath-antibody and other time periods were also not evaluated. Prior to secondary infection, the rear leg of 1 ferret demonstrated pitting edema distal to the popliteal lymph node; a second ferret developed bilateral pitting edema of the rear legs 2 weeks after secondary infection. The rear limb edema persisted and bilateral edema was evident in both animals at 24 weeks.

Multiple Infection

Further examination of the ferret immune response to filariasis was performed under conditions more applicable to exposure in endemic areas. Four ferrets were infected with 25 B. malayi third-stage larvae in the right rear leg every 2-4 weeks for seven exposures (Fig. 4). Ferrets became microfilaremic during the third or fourth month after initial infection. Peak microfilaremia varied from 40-3640 mf/ml. Clearance of circulating mf occurred at 143-318 days with a mean patency of 137 $\pm$ 46 days. An absolute eosinophilia was observed

Fig. 4. MICROFILAREMIA AND EOSINOPHILIA FOLLOWING MULTIPLE INFECTIONS WITH 25 B. MALAYI THIRD-STAGE LARVAE. Open bars represent total eosinophil counts and open dots represent numbers of circulating microfilariae. Arrows indicate infection with third-stage larvae. Cross (t) indicates termination of the experiment.


A7
A10
A9
A14
LOG 10 MICROFILARIAE PER ML BLOOD
TOTAL EOSINOPHILS PER MICROLITER BLOOD
DAYS POST INFECTION
DAYS POST INFECTION
40
120
200
280
1
2
3
1000
2000
3000
4000
5000
t

at patency in 3 of 4 ferrets and was highest (847-5940 eosinophils per microliter blood) near the time of microfilarial clearance (Fig.4). Following microfilarial clearance, IgG or IgM sheath-reactive antibody was identified in 2 of 4 ferrets (Ferret A10, 1:200 IgG and 1:40 IgM sheath-reactive antibody; Ferret A14, 1:10 IgM sheath-reactive antibody). Periodic examination of the rear legs identified 2 ferrets which developed edema of the right rear leg after microfilarial clearance. The edema waxed and waned, then persisted for the last three months of the experiment. Edema did not develop in the control leg injected similarily with PBS containing washed mosquito debris.

Intravenous Injection of Microfilariae

Ferrets infected with third-stage larvae are exposed to several larval developmental stages, as well as the adult parasites, and dissection of the host response to any particular developmental stage is naturally complicated in this type of infection. As an alternative experimental approach to infection with third-stage larvae, intravenous injection of *B. malayi* microfilariae was evaluated. This approach would facilitate experimental study by eliminating the time required for maturation of infection (prepatent period for *B. malayi* is approximately 3 months), confine host stimulation to a single life cycle stage, and permit experimental control of the infection by varying the timing and amount of microfilariae injected.

Primary Injection of Microfilariae

Following primary intravenous (i.v.) injection of 10^6 mf in 41 ferrets, a microfilaremia of 420-1340 mf/ml with a mean of 834 mf/ml was observed day 1 post-injection (i.e. approximately 10% of injected

microfilariae circulate in the peripheral blood on day 1 post-injection). Figure 5 shows the microfilaremia and eosinophil counts in 6 ferrets which represent the variations observed in ferrets injected intravenously with microfilariae. Persistence of microfilaremia was examined in 10 ferrets and mean microfilaremia $\pm$ SE on day 1, day 5-8 and day 20-27 was 867 $\pm$ 75, 710 $\pm$ 75 and 528 $\pm$ 75 mf/ml, respectively, which represents a slight, but statistically significant ($p < 0.05$) decrease in circulating microfilariae during the first 3-4 weeks following microfilarial injection. Seven ferrets cleared mf during the second month after injection and 3 cleared during the fourth and fifth months. The mean length of patency following the intravenous injection of microfilariae was 79 $\pm$ 15 days. During microfilaremia, circulating antibody reactive with the microfilarial sheath was rarely detected. However, IgG sheath-antibody was demonstrated in low titer (1:10) two weeks prior to mf clearance in one ferret. Following microfilarial clearance, all ferrets had detectable levels of IgG sheath-reactive antibody ranging in titer from 1:10 to 1:80; IgM sheath-reactive antibody (1:10) was observed in 1 ferret. Absolute eosinophil counts increased following the i.v. injection of mf and the highest levels occurred near the time of microfilarial clearance (Fig. 5); peak eosinophilia ranged from 945-2664 eosinophils per microliter blood. In addition, a transient neutrophilia was observed day 1 post i.v. injection of mf (Fig. 6).

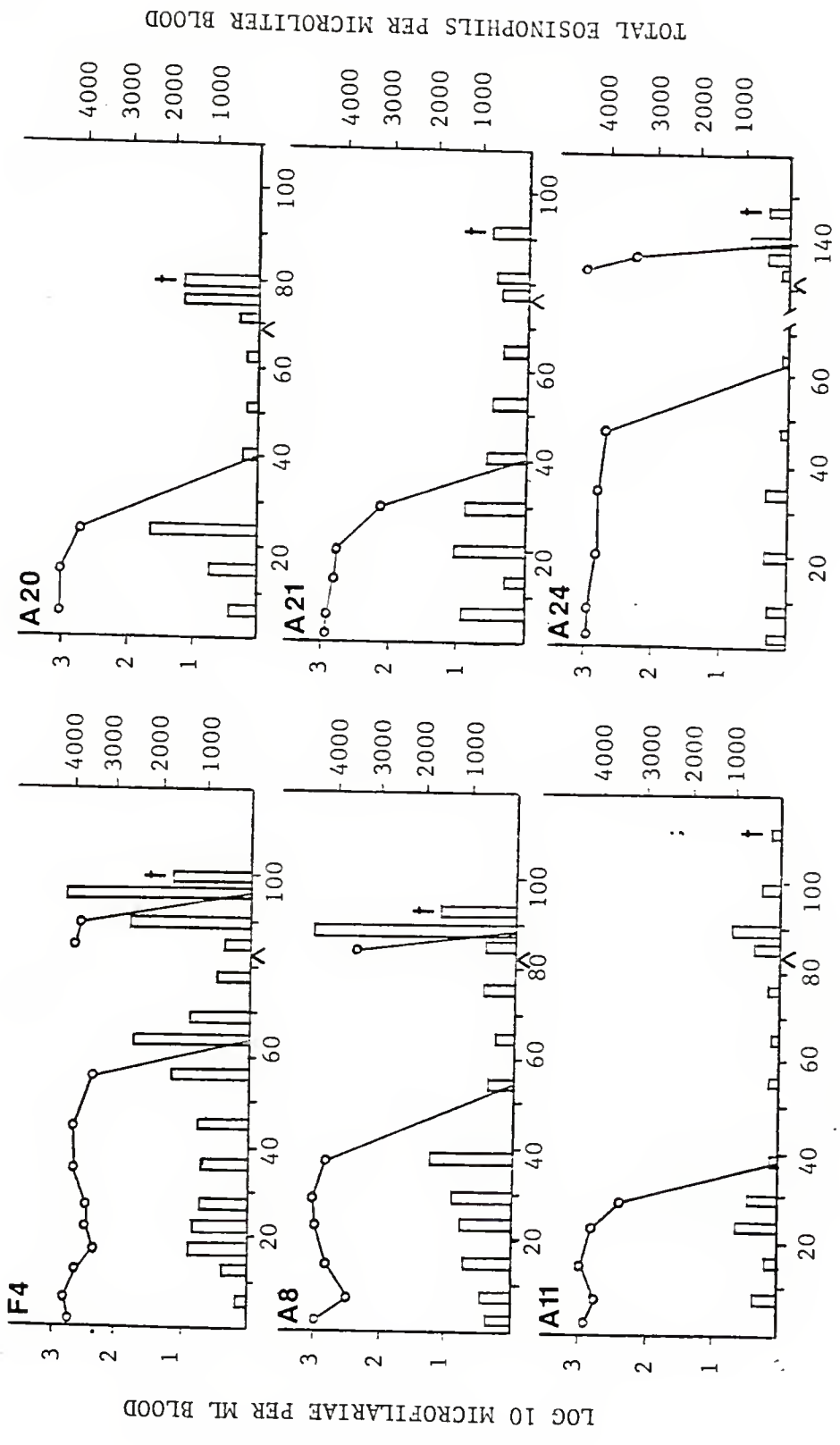
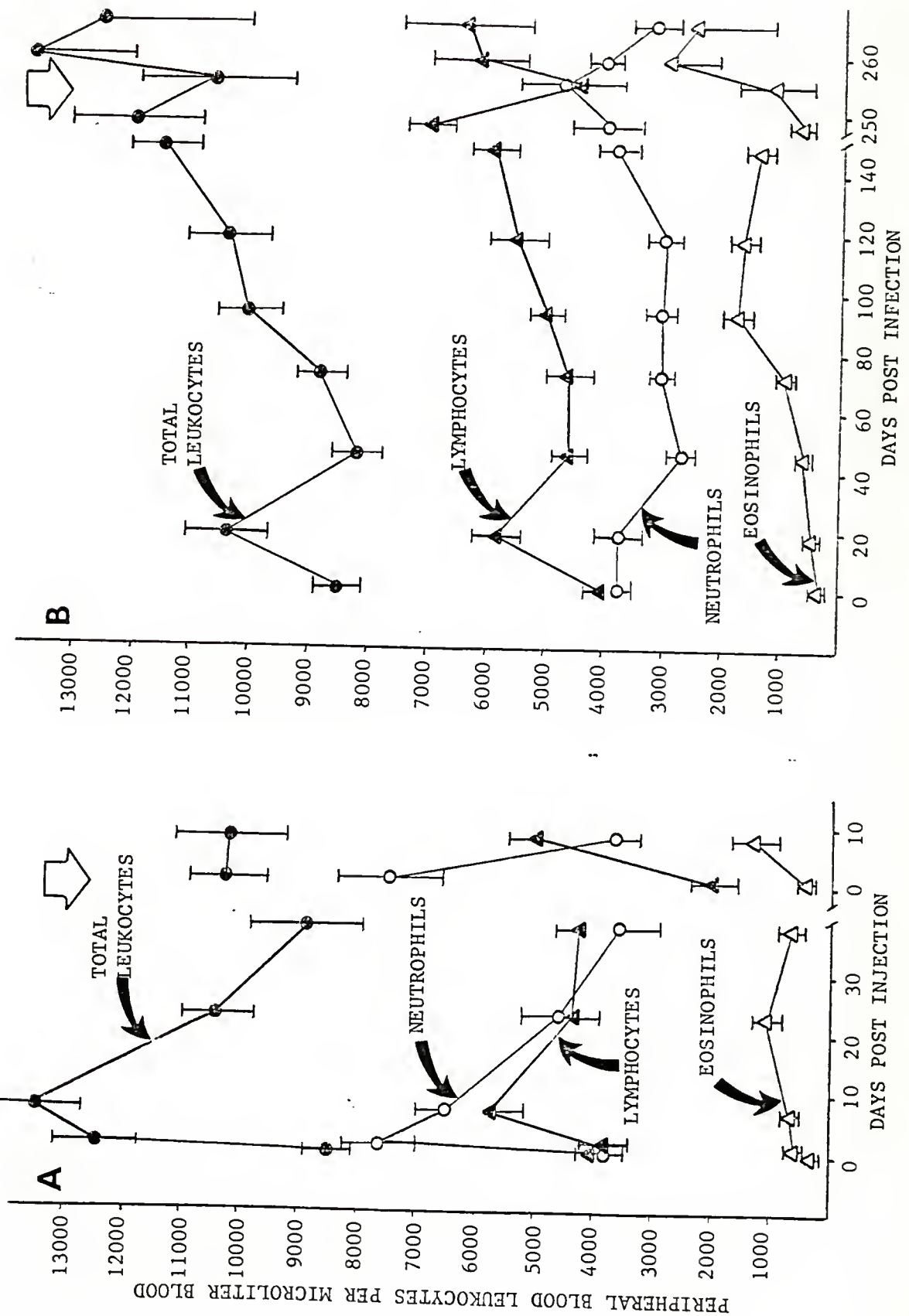


Fig. 5. MICROFILAREMIA AND EOSINOPHILIA FOLLOWING PRIMARY AND SECONDARY INTRAVENOUS INJECTION OF 10^6 B. MALAYI MICROFILARIAE. Open bars represent total eosinophil counts and open dots represent numbers of circulating microfilariae. Arrows indicate secondary i.v. injection of microfilariae. Cross (t) indicates termination of the experiment.

Fig. 6. TOTAL AND DIFFERENTIAL LEUKOCYTE COUNTS FOLLOWING INTRAVENOUS INJECTION OF 10^6 B. MALAYI MICROFILARIAE (10 FERRETS, A) AND FOLLOWING INFECTION WITH 100 B. MALAYI THIRD-STAGE LARVAE (16 FERRETS, B). Results expressed as mean ± SE. Total leukocytes ●—●, neutrophils ○—○, lymphocytes ▲—▲, and eosinophils △—△. Arrow indicates i.v. challenge with 10^6 B. malayi microfilariae (10 Ferrets; A. 4 Ferrets; B).


PERIPHERAL BLOOD LEUKOCYTES PER MICROLITER BLOOD
A
TOTAL LEUKOCYTES
NEUTROPHILS
LYMPHOCYTES
EOSINOPHILS
DAYS POST INJECTION
B
TOTAL LEUKOCYTES
LYMPHOCYTES
NEUTROPHILS
EOSINOPHILS
DAYS POST INFECTION

Secondary Injection of Microfilariae

Ferrets which had cleared intravenously injected mf and ferrets which had cleared mf produced by L3 infection were challenged with an i.v. injection of 10^6 *B. malayi* mf. Challenged ferrets remained amicrofilaremic or exhibited only a brief patency. Ferrets with IgG sheath-antibody titers of 1:40 or greater remained amicrofilaremic. Ferrets with IgG sheath-antibody titers of 1:20 or less, with one exception, had circulating mf day 1 post challenge injection (Table I). The period of microfilaremia was limited to 6 $\pm$ 2 days and 12 $\pm$ 5 days, respectively, for ferrets initially exposed to mf by L3 infection or by i.v. injection (Fig. 5). Following secondary challenge, ferrets exhibited a rapid increase in numbers of circulating eosinophils (Fig. 6). In addition, a transient decrease in the numbers of circulating lymphocytes was observed 1 day after secondary i.v. injection of microfilariae followed by a rapid return to pre-existing levels.

Hypersensitivity to Microfilarial Antigens

Ferrets which had cleared a secondary i.v. challenge of mf and ferrets following infection with third-stage larvae were skin tested with microfilarial antigens (Table II). Ferrets infected with third-stage larvae exhibited immediate hypersensitivity to microfilarial antigens which increased as the infection progressed. Ferrets which were skin tested following clearance of a secondary i.v. challenge of mf also exhibited immediate hypersensitivity, but were not as highly sensitive as those ferrets infected with L3. Ferrets did not exhibit obvious delayed hypersensitivity responses to

TABLE I.
MICROFILAREMIA FOLLOWING INTRAVENOUS CHALLENGE WITH 10^6 B.MALAYI MICROFILARIAE.

A. PRIMARY EXPOSURE TO MF BY I.V. MICROFILARIAL INJECTION.

FERRET[a]	SHEATH IgG TITER[c]	MF/ML BLOOD DAY 1
F4	-	500
A8	-	240
A25	-	1060
A24	1:20	960
A13	1:40	0
A11	$\geq$ 1:80	0
A20	$\geq$ 1:80	0

B. PRIMARY EXPOSURE TO MF BY L3 INFECTION.

FERRET[b]	SHEATH IgG TITER[c]	MF/ML BLOOD DAY 1
T2	-	780
T1	1:20	280
T8	1:20	140
T7	1:20	0

a. Ferrets were injected intravenously with 10^6 B. malayi mf and were amicrofilaremic for 2 to 68 days prior to i.v. challenge.

b. Ferrets were infected with 100 B. malayi L3 and were amicrofilaremic for 10 to 104 days prior to i.v. challenge.

c. Sheath-antibody measured by the indirect fluorescent antibody technique. Hyphen (-) indicates a titer < 1:10.

TABLE II.
IMMEDIATE CUTANEOUS HYPERSENSITIVITY TO MICROFILARIAL ANTIGENS.

A. FERRETS INJECTED INTRAVENOUSLY WITH 10^6 *B. MALAYI* MF.

NUMBER FERRETS	DAYS POST SECONDARY INJECTION	DIRECT SKIN TEST[a] 2.0ug	0.2ug	0.02ug	SALINE
7	7-15	17.1 ± 8.0	9.4 ± 3.3	2.4 ± 0.9	2.4 ± 0.9
2	29-54	ND[†]	25.1 ± 12.6	0.8 ± 0.0	0.8 ± 0.0

B. FERRETS INFECTED WITH 100 *B. MALAYI* L3.

NUMBER FERRETS[b]	DAYS POST INFECTION	DIRECT SKIN TEST[a] 2.0ug	0.2ug	0.02ug	SALINE
9	115-116	24.5 ± 4.6	7.9 ± 2.0	5.4 ± 1.2	6.1 ± 1.3
8	248-249	61.6 ± 4.8	30.4 ± 2.6	6.7 ± 2.0	5.1 ± 0.7
3	331-353	ND	49.7 ± 0.3	12.3 ± 10.4	2.9 ± 1.1

a. Ferrets were injected intradermally with 2.0ug, 0.2ug, and 0.02ug *B. malayi* microfilarial soluble somatic aqueous extract in 0.05 ml saline. Results expressed as mean area (mm) ± SE of skin test response 15 minutes after intradermal injection.

b. Ferrets that were skin tested on 115-116 days post infection were retested on days 248-249; 1 ferret was not retested.

†. ND = Not done.

microfilarial antigens 24-72 hours following intradermal testing; however, histologic examinations of the skin test sites were not performed. Sera obtained at the time of skin testing were examined in 72 hour Prausnitz-Kustner (PK) tests. Microfilarial-specific reaginic antibody was identified in each of 18 sera samples collected from ferrets infected with L3. Only 3 of 10 sera samples obtained from ferrets injected intravenously with mf exhibited microfilarial-specific reaginic activity; a 4 hour PK test was performed and a heat-stable (56°C for 2 hours) short-term homocytotropic antibody was not identified.

Inflammatory Responses

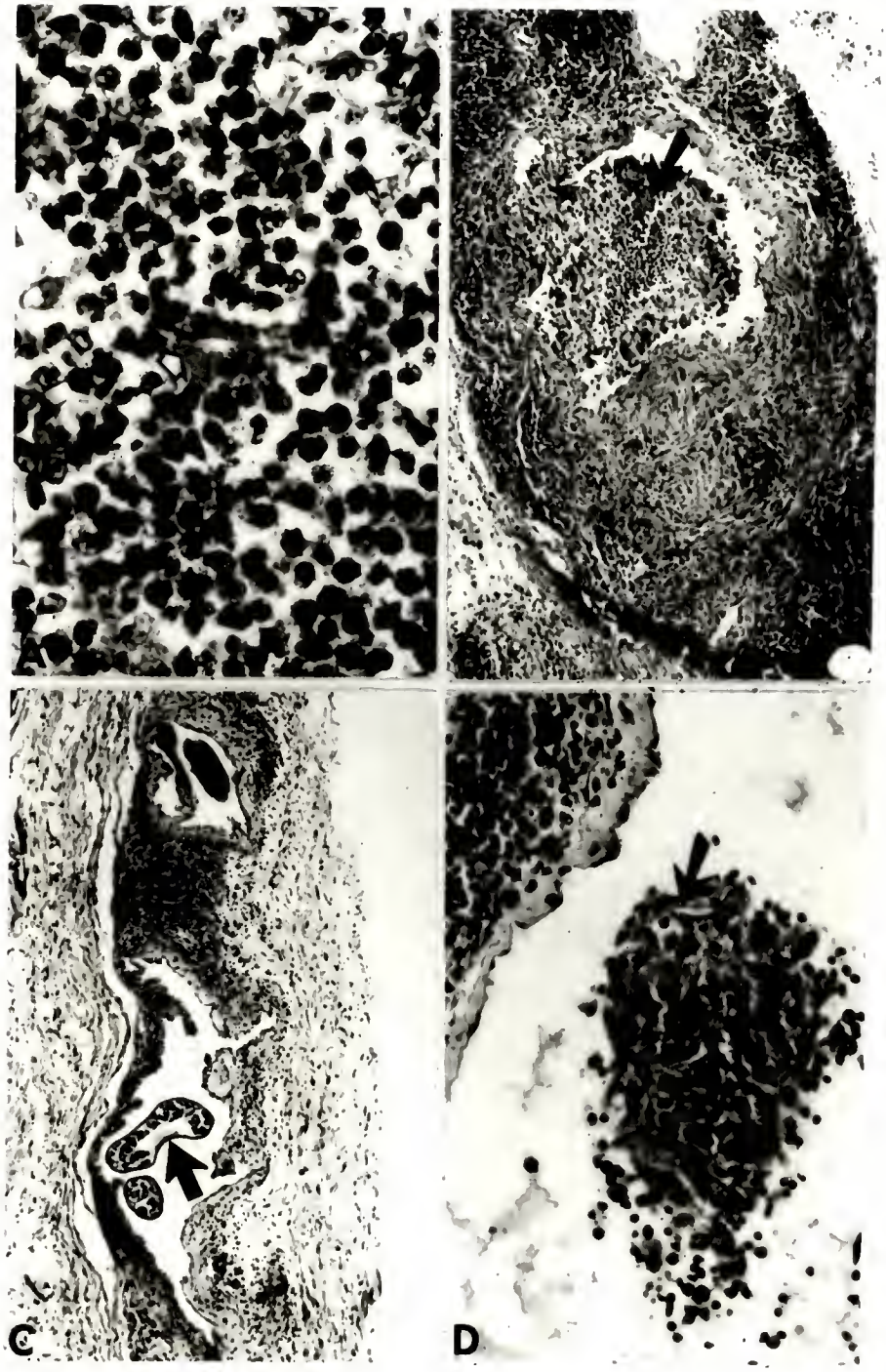
Initial histologic studies were performed on tissues collected at necropsy from ferrets infected with third-stage larvae (Fig. 7); necropsy was carried out within 3 months following the clearance of microfilariae. Lymphatic vessels which contained adult filariae were dilated and the draining lymph nodes enlarged. Histologic examination demonstrated adult worms within lymphatic lumens without adherent inflammatory cells on the cuticle of the worm; however, a cellular infiltrate with large numbers of lymphocytes and plasma cells was present within the walls of the lymphatic vessels which contained adult parasites. Within the lymphatic lumens, microfilariae without adherent cells were also observed, but degenerated microfilariae surrounded by acidophilic hyaline material (Splendore-Hoeppli deposits) were seen within thrombotic granulomas containing eosinophils, histiocytes, neutrophils and plasma cells. The inflammatory responses to microfilariae were not restricted to

Fig. 7. INFLAMMATORY CELL RESPONSES TO MICROFILARIAE WITHIN LYMPHATICS. A. A degenerating microfilaria in Splendore-Hoeppli material (arrow) within an eosinophilic abscess in a lymph node. Giemsa X630, AFIP No. 83-7009.

B. Lymphatic vessel with a thrombus and inflammation of the vessel wall; the thrombus contains Splendore-Hoeppli material (arrow) surrounded by eosinophils. Giemsa X60, AFIP No. 82-13715.

C. Diffuse lymphactic inflammation with thickened lymphatic walls and valves; a gravid female (arrow) within the lymphatic lumen is shown. Movat X60, AFIP No. 82-13715.

D. A dilated, inflamed lymphatic vessel containing a thrombus with microfilariae (arrow). H & E X250, AFIP No. 83-7003.


A
C
D

lymphatic vessels, but were observed within the draining lymph nodes, liver, lung, spleen and the gastrointestinal tract (Fig. 8); no lesions were identified within renal tissue. The lesions appeared grossly as white, well-demarcated, circular foci with diameters ranging from <1 mm to 2 mm and lesions were easiest to observe on the surface and cut section of the liver (Fig. 9). Microscopically these lesions were microabscesses with aggregations of eosinophils and smaller numbers of neutrophils, histiocytes and plasma cells. In the central portion of the lesion, acidophilic Splendore-Hoeppli (SH) substance was occasionally present and within some of the SH deposits a degenerated mf or microfilarial cast, 5-6 um in diameter, could be identified (Fig. 8A).

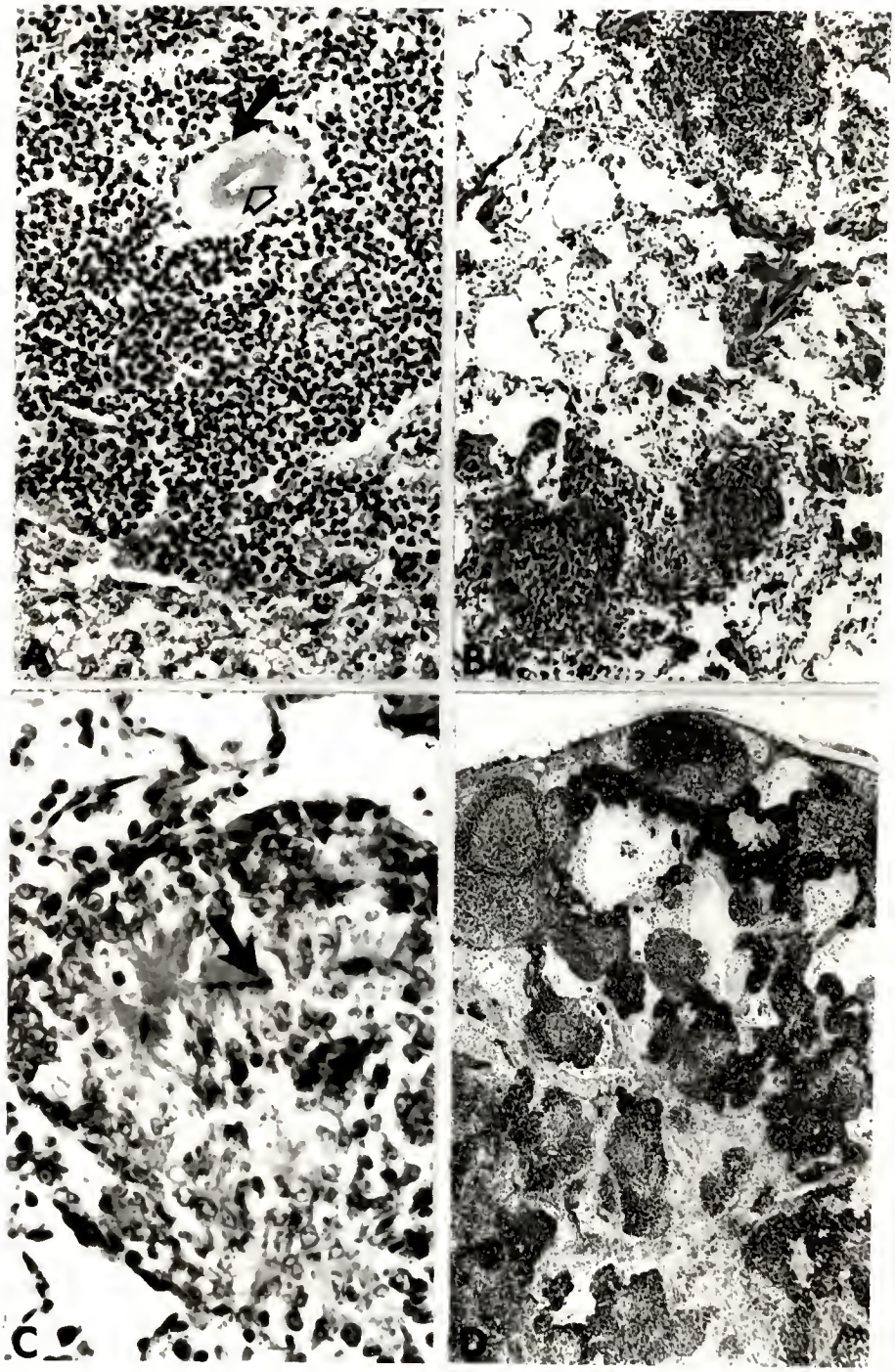
Concurrent with the histologic study of tissues collected from ferrets infected with third-stage larvae, ferrets which had cleared a secondary i.v. injection of microfilariae were necropsied and tissues similarily examined to determine if the intravenous injection of mf resulted in characteristic lesions of occult filariasis; immediately prior to necropsy, skin tests with microfilarial antigens were performed (results presented below). Four of 6 ferrets necropsied within 10 days following microfilarial clearance demonstrated the typical multifocal, white, well-demarcated surface and parenchymal liver lesions with diameters ranging from 1-2 mm, while 4 ferrets necropsied 11, 14, 18 or 21 days after microfilarial clearance did not exhibit gross liver lesions (Table III). Microscopically these gross liver lesions were microabscesses identical to those lesions observed in ferrets infected with third-stage larvae and were also found within the lung and spleen, but not within the kidney; other tissues were

Fig. 8. HISTOPATHOLOGIC RESPONSES TO MICROFILARIAE IN HEPATIC, PULMONARY AND LYMPHATIC TISSUES. A. Eosinophilic abscess in the liver surrounding Splendore-Hoeppli material (dark arrow) containing a degenerating microfilaria (white arrow). H & E X250, AFIP No. 82-13734.

B. Multifocal granulomas within the lung. H & E X100, AFIP No. 82-13711.

C. A degenerating microfilaria (arrow) encased in Splendore-Hoeppli material within a pulmonary granuloma. Giemsa X400, AFIP No. 82-13709.

D. Enlarged lymph node with hyperplastic germinal centers and dilated sinuses containing histiocytes and lymph. Giemsa X25, AFIP No. 82-13724.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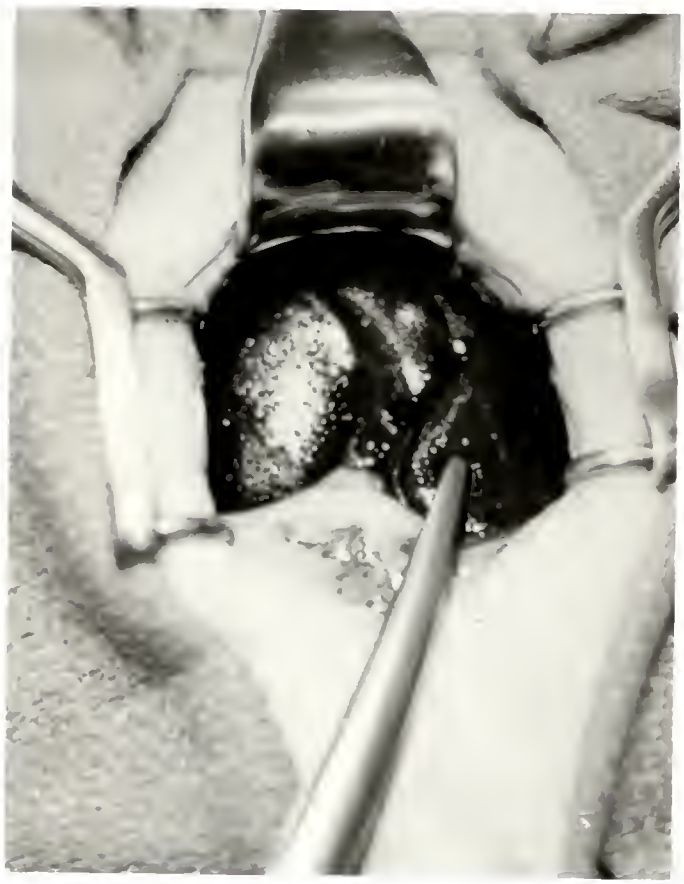


Fig. 9. LIVER WITH NUMEROUS CHARACTERISTIC SURFACE LESIONS OF OCCULT FILARIASIS AS VIEWED DURING EXPLORATORY LAPAROTOMY. Metzenbaum scissors point to a single lesion. Magnification; X1.

TABLE III.
SURFACE LIVER LESIONS AND IMMEDIATE HYPERSENSITIVITY TO MICROFILARIAL ANTIGENS.

FERRET[a]	DAYS POST SECONDARY CLEARANCE[b]	SURFACE LIVER LESIONS		HYPERSENSITIVITY[c]	
		GROSS	MICRO.	PK	DIRECT
A12	6	+	+	-	+
A18	8	-	-	-	-
A8	8	-	+	-	ND
F4	9	+	+	+	ND
A20	9	+	+	+	ND
A25	10	+	+	-	+
A21	11	-	-	-	-
A24	14	-	+	-	+
A11	18	-	-	+	ND
A13	21	-	-	-	ND

a. Ferrets were injected intravenously with 10^6 B. malayi mf and following clearance of circulating microfilariae were challenged intravenously with a second injection of 10^6 microfilariae.

b. Number of days ferrets were amicrofilaremic prior to necropsy.

c. Immediate hypersensitivity to microfilarial antigens was determined in 72 hour PK tests and direct skin tests using 0.2 ug B. malayi microfilarial soluble somatic aqueous extract in 0.05 ml saline. Skin test response determined 15 minutes after intradermal injection.

not examined. In addition, 2 ferrets without gross lesions had characteristic microscopic lesions of occult filariasis (Table III).

Liver Lesions and Immediate Hypersensitivity

Direct skin tests and Prausnitz-Kustner (PK) tests were used to correlate the presence of lesions characteristic of occult filariasis with immediate hypersensitivity to B. malayi microfilarial antigens in ferrets which had cleared a secondary i.v. injection of microfilariae (Table III). Prausnitz-Kustner tests were performed on sera collected from 10 ferrets at necropsy and direct skin tests were carried out in 5 of these 10 ferrets. Five of 7 ferrets necropsied within 14 days following clearance of microfilariae had gross or microscopic lesions characteristic of occult filariasis and exhibited either a positive direct skin test or PK skin test. The 2 ferrets which did not show immediate hypersensitivity to microfilarial antigens either by direct or PK skin tests did not exhibit gross or microscopic evidence of lesion formation. These results demonstrate a significant ($p < 0.05$) correlation between immediate hypersensitivity to microfilarial antigens and the presence of typical lesions of occult filariasis within 2 weeks following clearance of a secondary i.v. injection of microfilariae.

Development of the MK Body

Further examination of liver tissue obtained from ferrets infected with third-stage larvae and ferrets injected intravenously with microfilariae was carried out specifically to identify the cellular responses associated with the development of the

Meyers-Kouwenaar body. Microfilariae which were only slightly degenerated, as evidenced by intact nuclei, were observed within small inflammatory cell foci consisting of histiocytes and eosinophils. A narrow fringe of acidophilic Splendore-Hoeppli material surrounding the microfilariae was often apparent within these cellular responses (Fig. 10A). Serial sections of these foci demonstrated eosinophils adherent to the microfilariae and eosinophilic deposits on the microfilariae closely associated with these cells. Because the microfilariae within these cellular aggregates appeared only slightly degenerated, these cellular foci most likely represent early inflammatory responses in the formation of the MK body.

Microscopic examination of gross lesions showed a predominance of eosinophils. Meyers-Kouwenaar bodies observed within these eosinophilic abscesses varied from a degenerated microfilaria with a small fringe of Splendore-Hoeppli (SH) material to multiple layers of SH material radially arranged around a microfilarial remnant or a granular core which did not contain recognizable microfilarial structures (Fig. 10B). Immediately adjacent to the outer fringe of the SH material, eosinophils and eosinophil granules were observed; these eosinophils appeared to have degranulated directly onto the outer most fringe of the Meyers-Kouwenaar body and only the outer layers of the MK body stained intensely eosinophilic (Fig. 10B). Frozen liver sections containing MK bodies were examined for endogenous peroxidase. Intense endogenous peroxidase activity was obvious in surrounding eosinophils, in the outer layers of large MK bodies (Fig. 10C) and throughout the Splendore-Hoeppli material in small MK bodies.

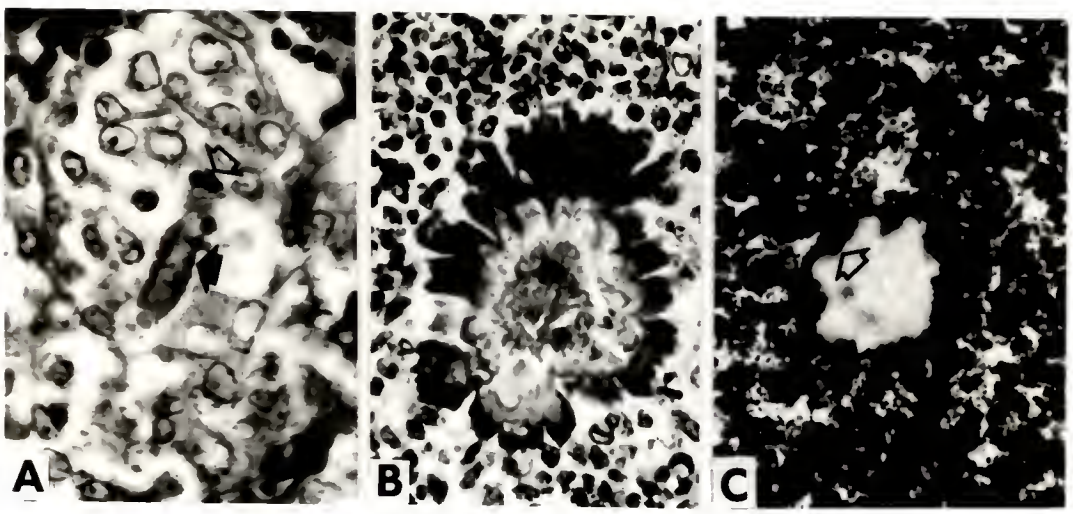


Fig. 10. LIGHT MICROSCOPY OF THE MEYERS-KOUWENAAR (MK) BODY. A. A small MK body demonstrating eosinophils (white arrow) and a fringe of acidophilic material on the surface of a microfilaria (dark arrow) in the liver; mononuclear cells, presumably Kupffer cells, surround the MK body. H & E X630.

B. A MK body with multiple layers of Splendore-Hoeppli material in the liver; eosinophils are adherent to the exterior edge of the MK body and only the outer layers of the MK body stain intensely eosinophilic. H & E X250.

C. Peroxidase staining of a MK body in a liver abscess; the outer layer of the MK body (arrow) and the surrounding eosinophils are heavily stained. H & E X250.

Transmission electron microscopy of the Meyers-Kouwenaar body confirmed both a layered, radial arrangement of SH material surrounding a degenerated mf and degranulated eosinophils at the outer most edge (Fig. 11A). The layers of SH material were heterogeneous and contained electron translucent and dense granular material. Within these layers were granules and apparently remnants of cellular organelles and nuclear material (Fig. 11B, C and D).

The granulomatous nature of the cellular inflammatory response to microfilariae was evident in the eosinophilic abscess stage of the MK body formation. Histiocytes and epithelioid cells surrounding these eosinophilic abscesses were often observed. In addition, granulomas containing foreign body giant cells and epithelioid cells were found surrounding only the MK body (Fig. 12) and occassionally, Splendore-Hoeppli material could be found within giant cells with no other associated cellular inflammatory response. Although these observations cannot establish with certainty the sequence of cellular inflammatory events in the development and the resolution of the MK body, the observations indicate that degranulating eosinophils contribute to the formation of the MK body and resolution of the MK body is accomplished within foreign body giant cells.

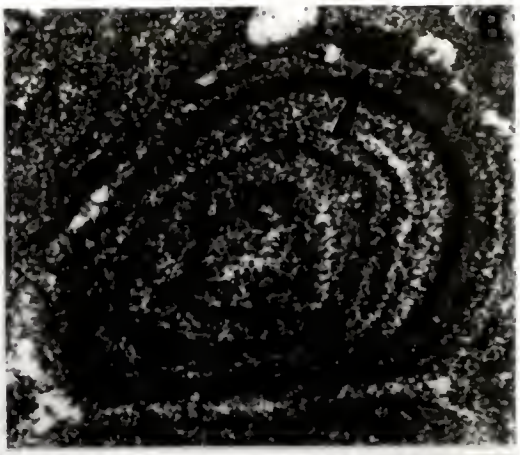
Microfilarial Clearance by Passive Transfer of Sera

Clearance of circulating microfilariae has been correlated with the presence of surface-reactive antibodies in filarial infections of animals (40,47,48) and Brugia malayi in man (34,37). To evaluate serum-mediated clearance of Brugia malayi microfilariae in the ferret, animals were injected intravenously with 10^6 B. malayi mf and immune

Fig. 11. ULTRASTRUCTURE OF THE MK BODY IN THE LIVER. A. Section showing a microfilaria (arrow) and layers of electron translucent and dense graular Splendore-Hoeppli material radiating apparently from the microfilarial sheath; the eosinophils at the outer edge are partially degranulated. X4,320.

B. The heterogeneous Splendore-Hoeppli material contains electron dense granules (arrow). X20,160.

C and D. At the periphery of the MK body, cell debris (C) and leukocyte granules (D) are incorporated into the Splendore-Hoeppli material. X8,190 and X25,725.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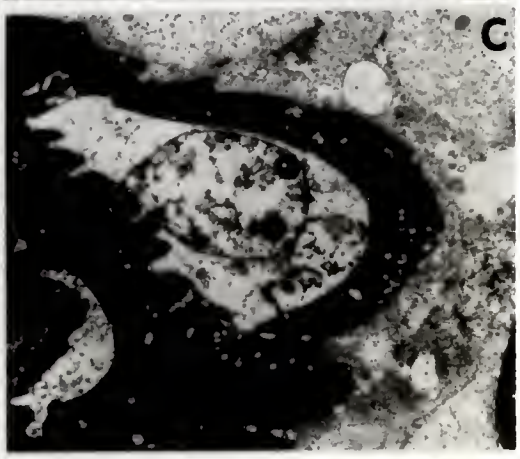
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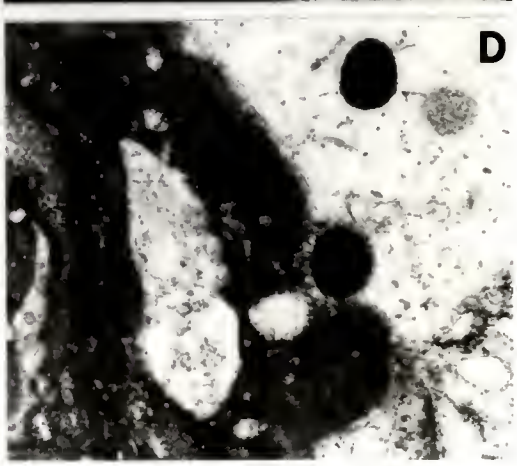
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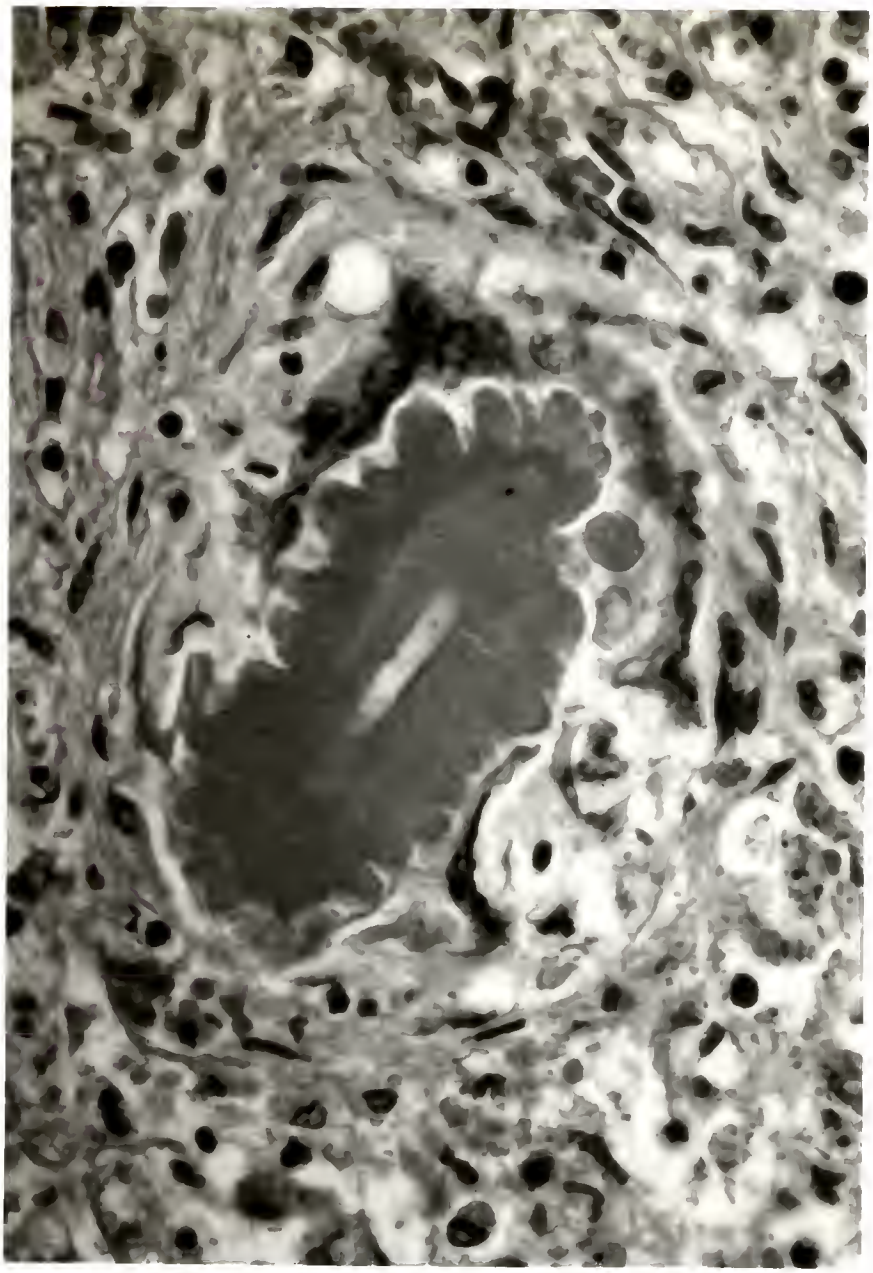


Fig. 12. GRANULOMA WITH A MULTINUCLEATED GIANT CELL SURROUNDING A MEYERS-KOUWENAAR BODY IN THE LIVER. H & E X480, AFIP No. 81-10419.

or normal ferret sera the following day (Table IV). Immune sera were collected from ferrets which had previously cleared injected mf; IgG and IgM sheath-antibody titer, IgE antibody titer to an extract of *B. malayi* mf, and microfilarial agglutination titer were measured prior to passive transfer. Sera with IgG sheath-antibody titers of 1:200 or greater and microfilarial agglutination titers of 1:64 cleared intravenously injected mf in less than 24 hours. Sera with an IgG sheath-antibody titer of 1:80 or with a microfilarial agglutination titer of 1:16 may have reduced numbers of circulating mf. Ferrets injected with sera containing high titers of microfilarial-specific reaginic antibody or IgM sheath-antibody, in the absence of IgG sheath-antibody, remained microfilaremic for the duration of the experiment as did ferrets injected with control sera.

To estimate the efficiency of immune sera in clearance of mf, ferrets were injected intravenously with decreasing amounts of serum known to clear mf (Table V). Ferrets injected with 1.0 to 2.0 ml of immune serum each cleared circulating mf, while 0.4 ml was only effective in clearing mf in 1 of 2 ferrets. Ferrets injected intravenously with 0.25 ml of immune serum remained microfilaremic, but the numbers of circulating microfilariae were significantly reduced ($p < 0.05$); mean microfilaremia $\pm$ SE pre- and 1 day post-injection were 865 ± 92 and 183 ± 49, respectively. Control ferrets injected with 0.25 to 1.0 ml normal sera remained microfilaremic for the duration of the experiment and did not demonstrate a significant reduction in microfilaremia; mean microfilaremia pre-injection was 912 ± 128 and 1 day post-injection was 692 ± 78 (Table V).

TABLE IV.
MICROFILARIAL CLEARANCE BY PASSIVE TRANSFER OF SERUM.

FERRET[a]	TITER OF INJECTED SERA: SHEATH[b] IgG	SHEATH[b] IgM	IgE[c]	AGG.[d]	VOL. INJECT.	MICROFILARIAE PER ML BLOOD: DAY 1[e]	DAY 2	DAY 8	DAY 22
A15	≥ 1:200	1:20	-	1:64	20 ML	1760	0	0	0
A19	≥ 1:200	1:20	-	1:64	20 ML	600	0	0	0
A23	≥ 1:200	-	1:1	ND†	20 ML	820	0	0	0
C13	≥ 1:200	1:90	-	1:64	2 ML*	800	0	0	0
C14	≥ 1:200	-	ND	1:16	4 ML	640	60	60	60
A26	1:80	ND	1:1	ND	20 ML	1060	580	580	80
C17	-	1:90	1:50	1:1	5 ML	500	720	440	730
C18	-	1:40	1:500	-	2 ML	660	560	500	180
A16	-	-	-	-	20 ML	660	980	860	1280
A22	-	-	-	-	20 ML	420	200	60	100
A24	-	-	-	-	20 ML	1100	720	800	640
A25	-	-	-	-	20 ML	1100	960	920	700

a. Ferrets were injected i.v. with 10^6 B.malayi mf and 24 hr. later injected with immune or normal sera; ferrets A16, A22, A24 and A25 received normal sera.

b. Sheath-antibody measured by the indirect fluorescent antibody technique. Hyphen (-) indicates a titer <1:10.

c. IgE antibody measured by 72 hour Prausnitz-Kustner tests using 0.2ug B. malayi mf soluble somatic aqueous extract in 0.05 ml saline. Hyphen (-) indicates a titer <1:1.

d. Microfilarial agglutination measured using 3000 B. malayi mf and dilutions of sera in PBS at a final volume of 0.2 ml. Incubation was carried out at room temperature for 30 min. with occasional shaking. Hyphen (-) indicates a titer <1:1.

e. Microfilaremia determined prior to serum injection.

* This sera subsequently titrated for clearance of microfilariae by passive transfer (see Table IV)

† ND = Not Done.

TABLE V.
MICROFILARIAL CLEARANCE BY PASSIVE TRANSFER OF SERUM: TITRATION OF SERUM.

A. PASSIVE TRANSFER OF IMMUNE SERUM.*

FERRET[a]	VOLUME INJECTED	MICROFILARIAE PER ML BLOOD			
		DAY 1[b]	DAY 2	DAY 8	DAY 22
C37	1.0 ML	760	0	0	0
C44	0.4 ML	700	0	0	0
C38	0.4 ML	1340	200	200	360
C39	0.25 ML	640	200	160	320
C40	0.25 ML	800	300	420	380
C45	0.25 ML	1060	170	120	120
C46	0.25 ML	960	60	80	40

B. PASSIVE TRANSFER OF NORMAL SERUM.

FERRET[a]	VOLUME INJECTED	MICROFILARIAE PER ML BLOOD			
		DAY 1[b]	DAY 2	DAY 8	DAY 22
C41	1.0 ML	1200	940	1060	640
C42	0.4 ML	540	460	320	80
C43	0.4 ML	1200	680	260	40
C47	0.25 ML	780	740	500	500
C48	0.25 ML	840	640	640	560

a. Ferrets were injected intravenously with 10 *B. malayi* mf and the following day received an i.v. injection of immune or normal sera.

b. Microfilaremia was determined prior to serum injection.

* Two milliliters of immune serum was previously shown to clear mf following passive transfer in Ferret C13 (see Table IV).

Sera collected from ferrets which had cleared injected mf were evaluated in passive transfer experiments to confirm serum-mediated clearance of mf established by infection with third-stage larvae; this experiment was carried out because mf harvested from the jird peritoneal cavity may have absorbed jird antigens onto the microfilarial sheath which could possibly be responsible for microfilarial clearance. Each of four ferrets were infected with 100 B. malayi third-stage larvae and were microfilaremic for 3-12 months prior to the i.v. injection of sera. Two ferrets were injected intravenously with 5 ml of immune sera exhibiting high titers of IgG sheath-antibody and microfilarial agglutination; two ferrets received control sera (Table VI). Ferrets injected with immune sera cleared or had reduced levels of circulating mf day 1 after injection, but circulating mf recurred 2-3 weeks following clearance; microfilaremia in control ferrets was not reduced. Taken together, these results indicate that clearance of mf can be mediated by a serum-dependent immune mechanism and that passive transfer of serum does not damage adult female filariae sufficiently to prevent the production of new microfilariae.

Specificity of Microfilarial Clearance

The specificity of microfilarial clearance by passive transfer of sera was evaluated using a filarial parasite of dogs, Dirofilaria immitis. Microfilariae were collected from the peripheral blood of one dog and 1.6×10^5 mf were injected intravenously into two ferrets; microfilaremia was measured the following day. One ferret received 3.0 ml of immune serum collected from a ferret which had cleared

TABLE VI.
MICROFILARIAL CLEARANCE BY PASSIVE TRANSFER OF SERUM IN FERRETS WITH ADULT INFECTION.

FERRET[a]	TITER OF INJECTED SERA: SHEATH[b] IgG	SHEATH[b] IgM	IgE[c]	AGG.[d]	VOL. INJECT.	MICROFILARIAE PER ML BLOOD: DAY 0[e]	DAY 1	DAY 3	DAY 17
A5	≥ 1:200	1:20	-	1:64	5 ML	2940	620	740	1340
T5	≥ 1:200	-	-	1:40	5 ML	720	0	0	40
T6	-	-	-	-	5 ML	340	200	560	420
T8	-	-	-	-	5 ML	1640	1380	2480	4140

a. Ferrets were infected with 100 B. malayi L3 and were microfilaremic for 3-12 months prior to i.v. injection of immune or normal sera; ferrets T6 and T8 received normal sera.

b. Sheath-antibody measured by the indirect fluorescent antibody technique. Hyphen (-) indicates a titer of <1:10.

c. IgE antibody measured by 72 hr. Prausnitz-Kustner tests using 0.2ug B.malayi microfilarial soluble somatic aqueous extract in 0.05 ml saline. Hyphen (-) indicates a titer <1:1.

d. Microfilarial agglutination measured using 3000 B. malayi mf and dilutions of sera in PBS at a final volume of 0.2 ml. Incubation was carried out at room temperature for 30 min. with occasional shaking. Hyphen (-) indicates a titer <1:1.

e. Microfilaremia determined prior to serum injection.

intravenously injected B. malayi mf; 1.0 ml of this immune serum was previously shown to clear 10^6 B. malayi mf. The control ferret received 3.0 ml of normal ferret serum. The i.v. injection of immune serum did not reduce levels of circulating D. immitis mf (Table VII).

Passive Transfer of Sephacryl-300 Fractionated Sera

Two normal and 2 immune sera with 1:200 or greater IgG sheath-antibody and 1:64 microfilarial agglutination titers were chromatographed on Sephacryl-300. Individual fractions (2.5 ml) were pooled to obtain major protein peaks and concentrated by ultrafiltration to the original loading volume (Fig. 13). Pooled fractions were examined for antibody specific for the microfilarial sheath by the indirect flourescent antibody and microfilarial agglutination tests. Selected fractions were then injected intravenously into microfilaremic ferrets to identify major protein peaks responsible for clearance of mf (Table VIII). The IgG-containing fraction of immune sera was the only fraction that cleared circulating mf; it was also the only fraction which agglutinated mf.

Opsonization of Microfilariae

The ability of intravenously injected B. malayi microfilariae to circulate following in vitro opsonization with immune or normal serum was determined; in vitro opsonization of microfilariae was carried out at room temperature in 30 ml PBS and dilutions of sera with occasional shaking. Four separate experiments were carried out using whole sera or the IgG-containing fraction of sera collected from

TABLE VII.
SPECIFICITY OF MICROFILARIAL CLEARANCE BY PASSIVE TRANSFER OF SERUM.

	TITER OF SERA			MICROFILARIAE PER ML BLOOD				
	SHEATH[b]							
FERRET[a]	IgG	IgM	AGG.[c]	DAY 1[d]	DAY 2	DAY 4	DAY 10	DAY 21
C15	≥ 1:200	1:90	1:64	104 ± 12	116 ± 22	144 ± 25	68 ± 16	16 ± 8
C16	-	-	-	172 ± 19	112 ± 8	60 ± 19	28 ± 5	28 ± 14

a. Ferrets were injected i.v. with 1.6×10^5 Dirofilaria immitis mf and 24 hr. later injected i.v. with 3 ml of immune or normal serum; ferret C16 received normal serum.

b. Sheath-antibody measured by the indirect fluorescent antibody technique. Hyphen (-) indicates a titer <1:10.

c. Microfilarial agglutination measured using 3000 B. malayi mf and dilutions of sera in PBS at a final volume of 0.2 ml. Incubation was carried out at room temperature for 30 min. with occasional shaking. Hyphen (-) indicates a titer <1:1.

d. Microfilaremia (mean of 5 counts ± std. err. mean) determined prior to the injection of serum.

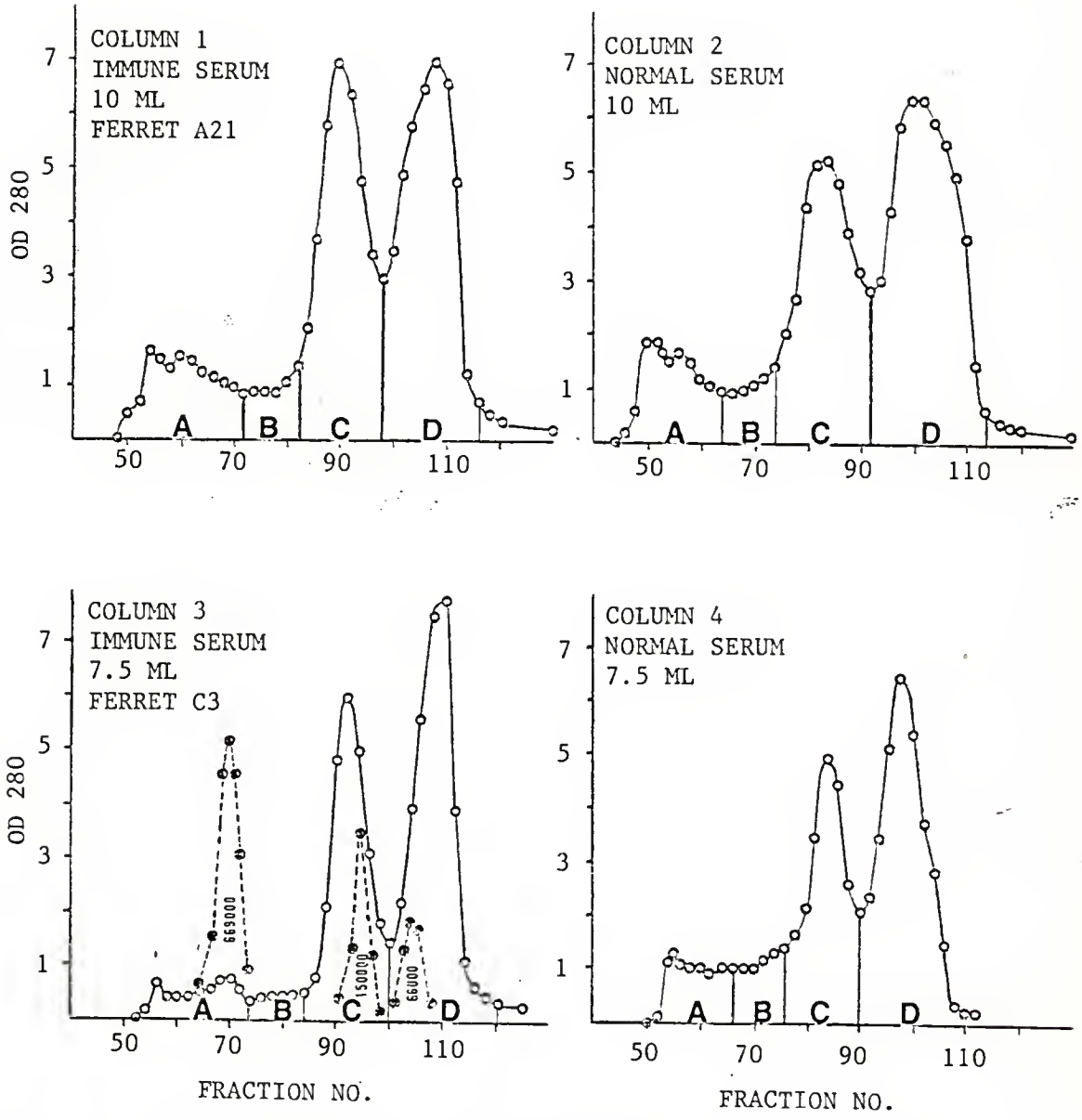


Fig. 13. GEL FILTRATION OF SERA THROUGH SEPHACRYL-300. Fractions (2.5 ml) were pooled as indicated and concentrated to original loading volume by ultrafiltration on YM-10 filters at 4° C. Molecular weight markers (Bovine thyroglobulin - 669000, alcohol dehydrogenase - 150000 and Bovine albumin - 66000) were filtered on Column 3. Open dots represent sera and closed dots represent molecular weight markers.

TABLE VIII.
MICROFILARIAL CLEARANCE BY PASSIVE TRANSFER OF SERUM FRACTIONS.

	FRACTIONS TRANSFERED				MICROFILARIAE PER ML BLOOD			
FERRET[a]	COL.[b]	FRACT.[b]	AGG. TITER[c]	VOL. INJECT.	DAY 1[d]	DAY 2	DAY 8	DAY 22
T10	1	C	1:20	7.5 ML	1140	0	ND	ND
T11	2	C	-	7.5 ML	820	540	740	800
C25	3	C	1:40	3.0 ML	810	0	0	0
C26	4	C	-	3.0 ML	1310	500	700	640
C27	3	A	-	6.0 ML	1030	210	270	180
C28	4	A	-	6.0 ML	460	160	370	260
C29	3	B	-	6.0 ML	1280	650	720	160
C30	3	D	-	6.0 ML	853	270	280	140

a. Ferrets were injected intravenously with 10^6 B. malayi mf and 24 hr. later were injected intravenously with sera fractions obtained by gel filtration through Sephacryl-300.

b. Sera fractions were pooled as indicated; see Fig. 13.
Column 1 = Immune serum; Ferret A21
Column 2 = Normal serum
Column 3 = Immune serum; Ferret C3
Column 4 = Normal serum

c. Microfilarial agglutination measured using 3000 B. malayi mf and dilutions of sera fractions in PBS at a final volume of 0.2 ml. Incubation was carried out at room temperature for 30 min. with occasional shaking. Hyphen (-) indicates a titer <1:1.

d. Microfilaremia determined prior to serum injection.

ferrets which had cleared intravenously injected microfilariae and sera collected from an amicrofilaremic ferret which received multiple infections with B. malayi third-stage larvae; control sera were obtained from normal ferrets (Table IX).

Intravenous injection of microfilariae opsonized in a 1:50 dilution of pooled immune serum (IPB) with a sheath-IgG antibody titer $\geq$ 1:200 and no detectable IgM sheath-antibody failed to circulate or circulated at markedly reduced levels. Microfilaremia following i.v. injection of microfilariae opsonized in a 1:200 or 1:800 dilution of this same immune serum pool was not significantly different from the microfilaremia established by i.v. injection of control microfilariae opsonized in normal sera (Exp. 1 and 2, Table IX). Microfilariae opsonized in a 1:40 dilution of an IgG-containing fraction of immune serum (A21 IgG) with a sheath-IgG antibody titer $\geq$ 1:200 also circulated at reduced levels (Exp. 3, Table IX). In contrast, the in vitro opsonization of microfilariae in a 1:20, 1:100 or 1:500 dilution of immune sera containing high titers of sheath-IgM antibody (A10, 1:40), circulated at levels comparable to control microfilariae opsonized with normal serum (Exp. 4, Table IX).

A complement fixation assay was subsequently performed to measure complement fixing activity following in vitro opsonization of microfilariae. (Fig. 14). Microfilariae opsonized in sera containing sheath-reactive IgM antibody (A10, 1:40) fixed more complement than did microfilariae opsonized in sera containing only IgG sheath antibody. However, the ability of opsonized mf to fix complement was, by itself, not sufficient to prevent microfilarial circulation as shown in the preceding experiments (Table IX).

TABLE IX
MICROFILAREMIA FOLLOWING INTRAVENOUS INJECTION OF OPSONIZED MICROFILARIAE.

EXP. NO.	FERRET[b]	OPSONIZATION OF MF[a] NO. MF	SERA	DILUTION	MICROFILARIAE PER ML BLOOD DAY 1	DAY 8-10
1	A33	10^6	IPB[c]	1:50	20	0
	A34	10^6	NFS[d]	1:5	1140	1200
2	A39	$5X10^5$	IPB	1:50	0	0
	A42	$5X10^5$	IPB	1:200	580	420
	A43	$5X10^5$	IPB	1:800	660	480
	A41	$5X10^5$	NFS	1:50	500	460
3	T13	$5X10^5$	A21 IgG[e]	1:40	20	0
	T12	$5X10^5$	NFS IgG	1:40	720	400
4	C20	10^6	A10[f]	1:20	660	780
	C22	10^6	A10	1:100	440	ND
	C23	10^6	A10	1:500	840	ND
	C21	10^6	NFS	1:20	900	680

a. Opsonizations of microfilariae were carried out at room temperature in 30 ml PBS and dilutions of immune or normal sera with occasional shaking.

b. Ferrets were injected intravenously with opsonized mf and 24 hr. later the numbers of circulating microfilariae were determined.

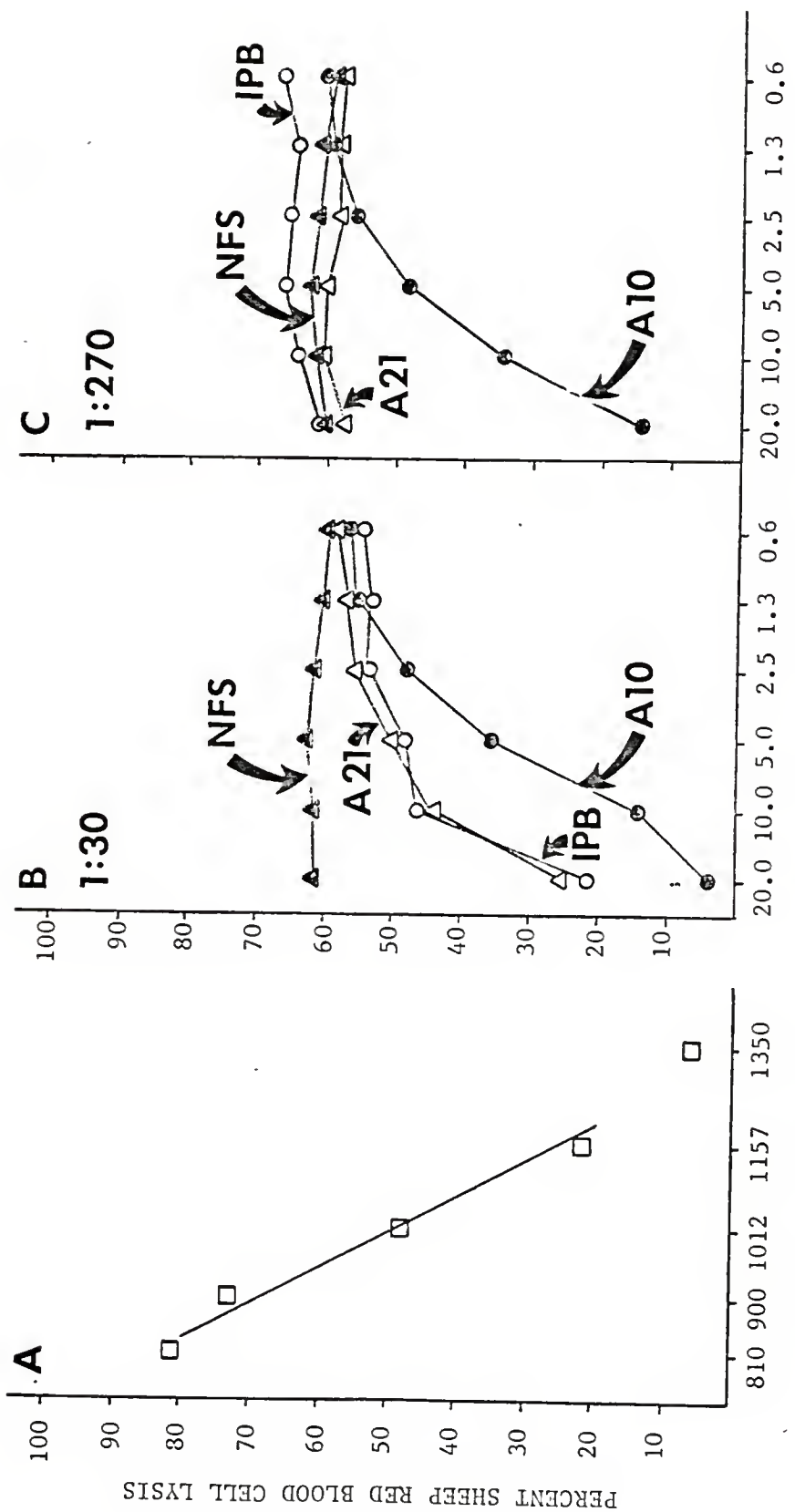
c. IPB- immune sera pool obtained from three ferrets which cleared a secondary i.v. injection of microfilariae. Sheath IgG $\geq$ 1:200 and Sheath IgM <1:10.

d. NFS- normal ferret serum.

e. A21 IgG- IgG containing fraction of immune serum obtained by chromatographic separation on a Sephacryl-300 column; see Fig. 13. Immune sera was collected from Ferret A21 following clearance of a secondary intravenous injection of microfilariae. Sheath IgG $\geq$ 1:200 and Sheath IgM <1:10.

f. A10- immune sera obtained from Ferret A10 which had cleared circulating microfilariae established by multiple infections of *B. malayi* third-stage larvae. Sheath IgG 1:200 and Sheath IgM 1:40.

Fig. 14. TITRATION OF COMPLEMENT ACTIVITY IN NORMAL FERRET SERUM (A) AND COMPLEMENT FIXATION FOLLOWING INCUBATION WITH OPSONIZED MICROFILARIAE (B AND C). A 1:900 dilution of normal ferret serum was used as the source of complement in B and C. Microfilariae in B were opsonized with a 1:10 or 1:30 dilution of normal or immune sera, respectively. Microfilariae in C were opsonized in a 1:10 or 1:270 dilution of normal or immune sera, respectively. IPB ○—○, immune sera pool obtained from three ferrets which cleared a secondary i.v. injection of microfilariae: sheath-IgG $\geq$ 1:200 and sheath-IgM $<$1:10. A21 △—△, immune sera collected from Ferret A21 following clearance of a secondary i.v. injection of microtilariae: sheath-IgG $\geq$ 1:200 and sheath-IgM $<$1:10. A10 ●—●, immune sera obtained from Ferret A10 which had cleared circulating microfilariae established by multiple infections with third-stage larvae: sheath-IgG 1:200 and sheath-IgM 1:40. NFS ▲—▲, normal ferret sera.


A
B
C
1:30
1:270
NFS
IPB
A21
A10
PERCENT SHEEP RED BLOOD CELL LYSIS
RECIPROCAL DILUTION OF NORMAL FERRET SERUM
NUMBER OF MICROFILARIAE PER TUBE X10^{-3}

Induction of Lesions of Occult Filariasis

Concurrent with the study of microfilarial clearance, peripheral blood eosinophil counts were determined and liver tissue was examined for lesions characteristic of occult filariasis (Table X). Six ferrets injected intravenously with 0.4 - 20.0 ml of immune sera cleared intravenously injected mf by day 1 after serum transfer; the immune sera used for transfer were obtained from 3 ferrets which had cleared injected mf and had essentially no IgE antibody to antigens of mf by PK tests, titer $\leq$ 1:1, but did exhibit high titers of IgG antibody to the microfilarial-sheath (Table IV). No increase in peripheral blood eosinophils and no gross or microscopic liver lesions were observed at biopsy 1 week after serum transfer, or at necropsy at 3 weeks (Table X, Immune clearance).

Five ferrets which remained patent following i.v. injection with 0.25 - 0.4 ml (subclearance dose) of a single immune sera containing no microfilarial-specific reaginic antibody (Table IV) and 12 control ferrets injected with 0.25 - 20 ml of normal sera were examined for eosinophilia and gross liver lesions. One of 5 ferrets injected with a subclearance dose of immune serum exhibited approximately 50 liver lesions 1 week after serum transfer and 2 of the remaining 4 ferrets had 5 liver lesions at 3 weeks; a marginal increase in peripheral blood eosinophilia was observed at 3 weeks. Two of the 12 control ferrets had a few lesions (<5) at 1 week and 8 of 10 ferrets examined at 3 weeks exhibited a mean of 80 $\pm$ 60 surface liver lesions; an absolute eosinophilia, but no significant decrease in microfilaremia, was observed at 3 weeks (Table X, Immune subclearance and Normal).

TABLE X.
SUMMARY OF MICROFILAREMIA, EOSINOPHILIA AND LESIONS OF OCCULT FILARIASIS FOLLOWING PASSIVE TRANSFER OF SERUM.

A. ONE WEEK.

SERA USED FOR PASSIVE TRANSFER	MICROFILAREMIA[e]	EOSINOPHILIA[f]	NO. FERRETS WITH LIVER LESIONS/NO. FERRETS
NORMAL[a]	638 ± 98	460 ± 61	2/12
IMMUNE CLEARANCE[b]	0 ± 0	662 ± 96	0/6
IMMUNE SUBCLEARANCE[c]	196 ± 59	652 ± 200	1/5
IMMUNE PK POSITIVE[d]	609 ± 85	1137 ± 153	6/11

B. THREE WEEKS.

SERA USED FOR PASSIVE TRANFSER	MICROFILAREMIA[e]	EOSINOPHILIA[f]	NO. FERRETS WITH LIVER LESIONS/NO. FERRETS
NORMAL[a]	547 ± 113	1261 ± 392	8/10
IMMUNE CLEARANCE[b]	0 ± 0	489 ± 92	0/6
IMMUNE SUBCLEARANCE[c]	244 ± 69	836 ± 270	3/5
IMMUNE PK POSITIVE[d]	393 ± 102	2103 ± 606	9/10

a. Normal sera- obtained from 6-12 month old ferrets (volume transferred; 0.25-20.0 ml).

b. Immune clearance sera- obtained from 3 ferrets which cleared intravenously injected mf and exhibited high titers of sheath antibody, but essentially no reaginic antibody to microfilarial antigens (volume transferred; 0.4-20.0 ml).

c. Immune subclearance sera- obtained from a single ferret which cleared intravenously injected mf and exhibited a high titer of sheath antibody, but no reaginic antibody to microfilarial antigens (volume transferred; 0.25-0.4 ml).

d. Immune PK positive sera- obtained from 7 ferrets infected with third-stage larvae which had cleared mf and from a single ferret which cleared intravenously injected mf. These sera exhibited low sheath antibody, but high titers of microfilarial specific reaginic-antibody (volume transferred; 2.0-20.0 ml).

e. Mean microfilaremia per ml blood ± SE.

f. Mean eosinophilia per per microliter blood ± SE.

Sera collected from 7 ferrets infected with third-stage larvae which had cleared mf, and from 1 ferret injected i.v. with mf, were individually evaluated for passive induction of filarial lesions; these sera had < 1:100 titers of antibody to microfilarial sheath by IFAT and titers of 1:1 to 1:500 microfilarial specific IgE antibody by PK test. Two to 20.0 ml of sera were injected into 11 ferrets 2 days before or 1 day after an i.v. injection of mf. One week after injection of mf an absolute eosinophilia was observed and 6 of 11 ferrets had a mean of 39 $\pm$ 16 surface liver lesions which represents a significant ($p < 0.05$) increase, from control ferrets, in both numbers of ferrets which showed liver lesions and numbers of surface lesions; 9 of 10 ferrets examined at 3 weeks had 139 $\pm$ 69 liver lesions which was not significantly different from control ferrets. These ferrets remained microfilaremic through the duration of the experiment and a significant reduction of microfilaremia was not observed (Table X, Immune PK positive). These results suggest that passive transfer of sera, containing high titers of microfilarial-specific reaginic antibody, may sensitize ferrets for induction of lesions characteristic of occult filariasis.

Absolute peripheral blood eosinophil counts and the number of surface liver lesions at 1 and 3 weeks after i.v. injection of mf were compared in all groups (Fig. 15). There was a significant linear correlation ($p < 0.05$) between the numbers of blood eosinophils and the number of surface liver lesions at 1 and 3 weeks ($r = 0.6426$ and $r = 0.8802$, respectively). Direct skin tests with extracts of mf and PK tests on sera were performed on ferrets injected with control sera at 1 and 3 weeks after i.v. injection of mf. Microfilarial-specific

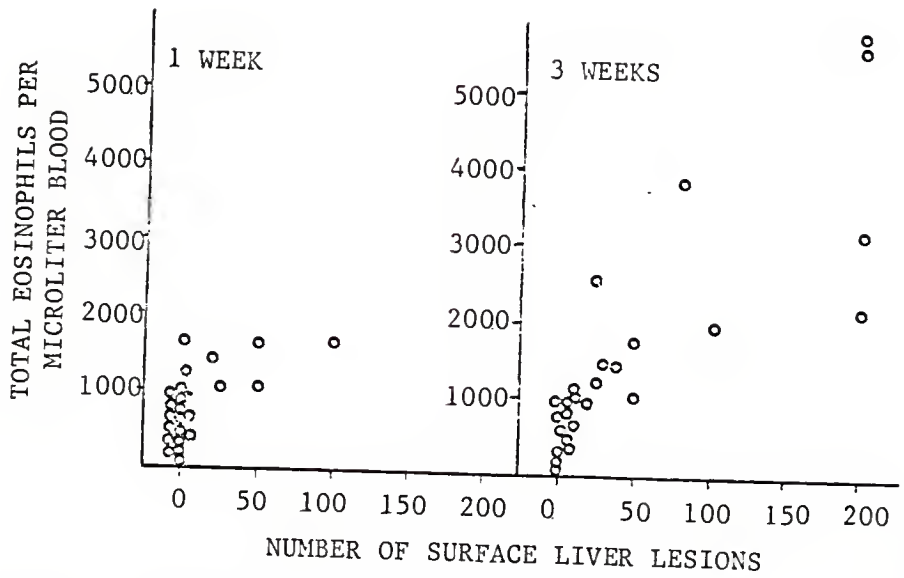


Fig. 15. LEVELS OF EOSINOPHILS AS A FUNCTION OF SURFACE LIVER LESIONS IN FERRETS INJECTED INTRAVENOUSLY WITH 10^6 B. MALAYI MICROFILARIAE AND IMMUNE OR NORMAL SERA. The absolute numbers of blood eosinophils are plotted against the numbers of surface liver lesions. There is a significant linear correlation ($p < 0.05$) between the numbers of blood eosinophils and the numbers of surface liver lesions at 1 and 3 weeks ($r = 0.6426$ and $r = 0.8802$, respectively).

reaginic antibody was not detected by PK tests at either time period. Direct skin tests at 1 week in the 7 ferrets tested did not demonstrate immediate cutaneous hypersensitivity, but 2 of 3 ferrets tested at 3 weeks exhibited a positive direct skin test and had 80 and 500 surface liver lesions at this time; the third ferret exhibited 25 surface liver lesions.

In summary, these results indicate that (1) the rapid clearance of microfilariae by passive transfer of sera does not lead to the formation of the characteristic lesions associated with occult infection, (2) the passive transfer of sera containing high titers of microfilarial-specific reaginic antibody results in accelerated development of lesions and (3) the extent of blood eosinophilia reflects the degree of surface liver lesions typical of occult filariasis.

B. malayi Microfilariae in T. spiralis Infected Ferrets

To determine if reaginic antibody with specificity for microfilarial antigens was required for the development of lesions characteristic of occult filariasis, ferrets were infected with T. spiralis to induce a peripheral blood eosinophilia in the absence of immediate hypersensitivity to B. malayi microfilarial antigens. Following oral infection with 500-700 T. spiralis larvae in 17 ferrets, an absolute eosinophilia was observed on day 8 and persisted through day 30 post infection. Peak eosinophilia ranged from 1140-9600 eosinophils per microliter blood and occurred 3-4 weeks after infection (Fig. 16). On day 30 following oral infection with T. spiralis, the ferrets were injected intravenously with 10^6 B. malayi

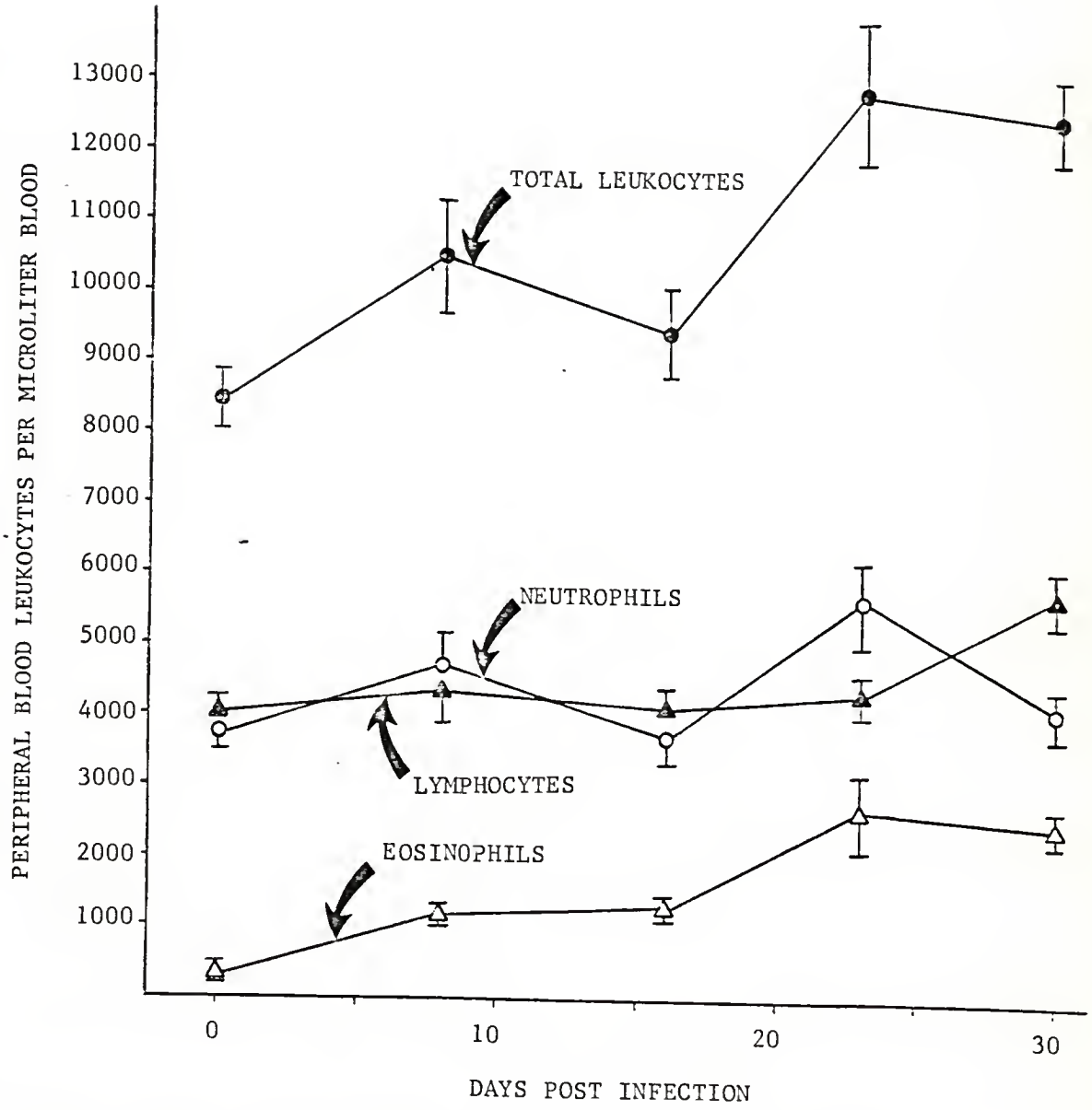
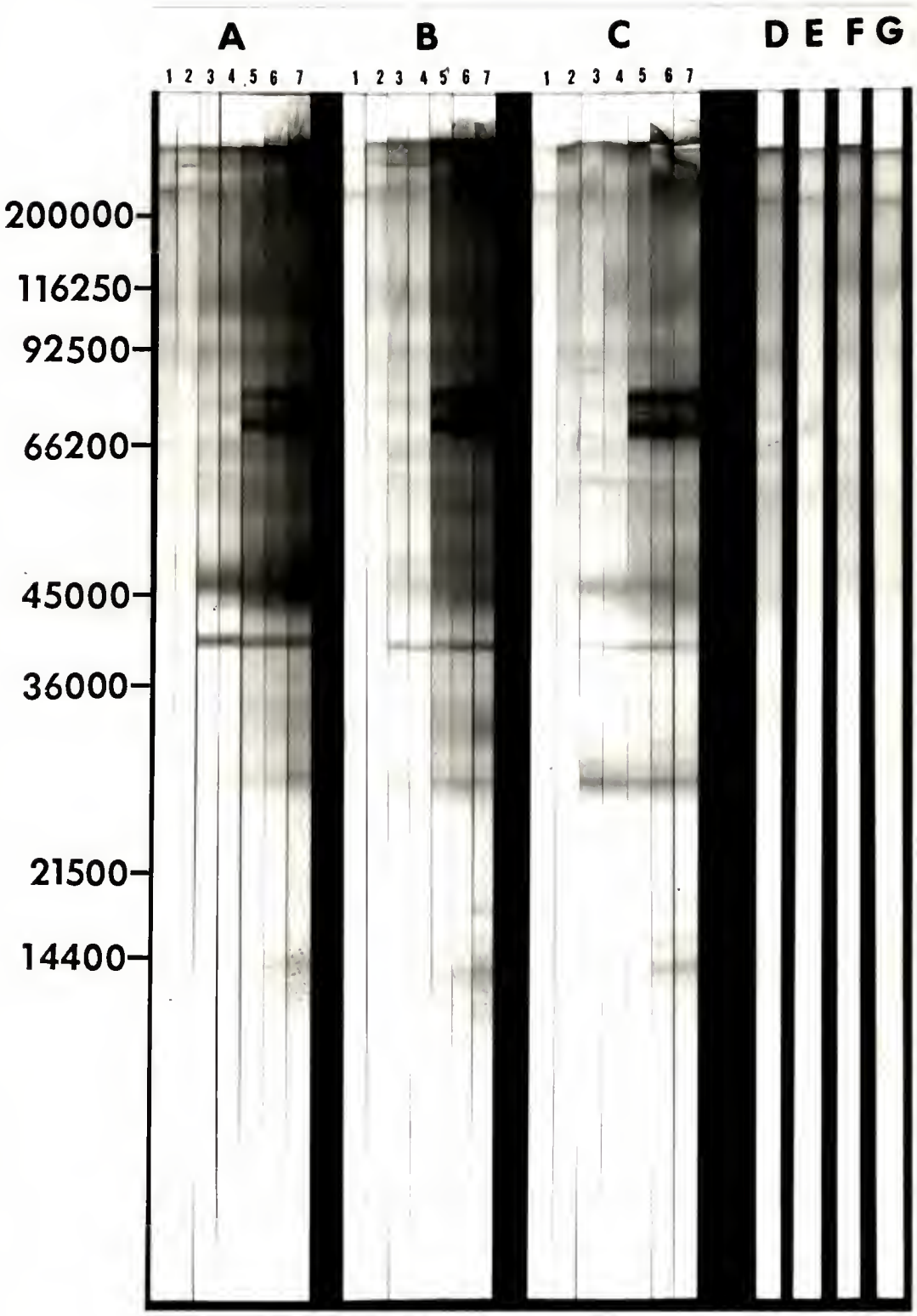


Fig. 16. TOTAL AND DIFFERENTIAL LEUKOCYTE COUNTS FOLLOWING ORAL INFECTION WITH 500-700 T. SPIRALIS LARVAE. Results are expressed as mean ± SE of 16 ferrets. Total leukocytes ●—●, neutrophils ○—○, lymphocytes ▲—▲, and eosinophils △—△.

microfilariae. Each of 5 ferrets infected with T. spiralis and then injected intravenously with B. malayi microfilariae exhibited $\geq$ 500 surface liver lesions characteristic of occult filariasis on day 4-7 post-injection. Two control ferrets that were infected with T. spiralis, but were not injected with microfilariae, did not demonstrate liver pathology. Direct skin test performed on day 4-7 following i.v. injection of microfilariae demonstrated no immediate hypersensitivity to microfilarial antigens. In addition, 4 and 72 hour PK tests using sera collected before i.v. injection of microfilariae and 7 days after injection showed no hypersensitivity to microfilarial antigens; at 3 weeks following i.v. injection of microfilariae, low levels of reaginic antibody specific for microfilarial antigens were detected. Sera collected from ferrets 30 days following oral infection with T. spiralis were also examined to detect cross-reactive antibody to B. malayi microfilarial antigens by Western Blot analysis (Fig. 17). Ferrets infected with Trichinella exhibited little antibody to B. malayi microfilarial antigens when compared to humoral responses of ferrets infected with B. malayi third-stage larvae 30 days after the appearance of circulating microfilariae (Fig. 17) or 30 days after the i.v. injecton of microfilariae (data not shown).

Two ferrets infected with T. spiralis were injected with a single immune serum containing a 1:500 titer of microfilarial-specific reaginic antibody on day 29 post oral infection. The following day, each ferret received 10^6 B. malayi microfilariae intravenously. These ferrets exhibited 10 and 200 surface liver lesions and had a positive immediate hypersensitivity skin test to microfilarial antigens. These

Fig. 17. WESTERN BLOT ANALYSIS OF THE ANTIBODY RESPONSES OF FERRETS INFECTED WITH B. MALAYI OR T. SPIRALIS TO MICROFILARIAL ANTIGENS. Lanes A, B and C represent the antibody responses of 3 ferrets [Ferret A1, A4 and A5, respectively; (see Fig. 3)] infected with 100 B. malayi third-stage larvae to B. malayi microfilarial antigens during infection; lanes 1-7 represent sera samples obtained at 0, 31, 63, 73, 95, 120 and 150 days post-infection, respectively. Lanes D, E, F and G represent the antibody responses of 4 ferrets infected with 500-700 T. spiralis larvae to B. malayi microfilarial antigens at 30 days post-infection. Numbers at the left margin represent molecular weight standards.


A
B
C
D E F G
1 2 3 4 5 6 7
1 2 3 4 5 6 7
1 2 3 4 5 6 7
200000
116250
92500
66200
45000
36000
21500
14400

results suggested (1) that presensitization of Trichinella infected ferrets with microfilarial-specific reaginic antibody did not lead to increased numbers of liver lesions, (2) that tissue (skin) mast cells were not degranulated as a result of Trichinella infection or the i.v. injection of microfilariae and (3) that saturation of the Fc receptors on mast cells with Trichinella-specific IgE antibody did not occur.

To determine the effect of rapid microfilarial clearance on the extent of liver pathology in ferrets infected with T. spiralis, 2 ferrets were cleared of circulating mf on day 1 by passive transfer of sera. On day 4, exploratory laparotomy and wedge liver biopsy were performed. Surface liver lesions characteristic of occult filariasis were observed; but, the numbers of lesions (30 and 100) were less than those observed in three control ferrets ($\geq$ 500) infected with T. spiralis which did not receive sera. Each of these animals was necropsied 17 days after the intravenous injection of microfilariae. The two ferrets cleared of circulating microfilariae on day 1 exhibited no gross lesions on the surface or within the parenchyma of the liver, while the control ferrets demonstrated mean surface liver lesions of 367 $\pm$ 133. Extensive histologic examination of the liver from the two ferrets rapidly cleared of circulating microfilariae identified a single granuloma containing multinucleated giant cells surrounding Splendore-Hoeppli material.

To determine the timing and sequence of cellular responses to microfilariae and the development of the MK body following i.v. injection of microfilariae in Trichinella infected ferrets, 4 ferrets were infected with 500-700 T. spiralis larvae and 30 days post infection were injected with 10^6 B. malayi microfilariae. An

exploratory laparotomy and wedge liver biopsy were performed on these ferrets on day 1 post i.v. injection of microfilariae; ferrets were then necropsied the following day. Meyers-Kouwenaar bodies were not observed at either time period. However, small aggregates of predominently neutrophils and mononuclear cells with a few eosinophils were observed on day 1 and an increased number of eosinophils and mononuclear cells observed on day 2 following the i.v. injection of microfilariae.

DISCUSSION

This investigation has examined the immunobiology and pathology of Brugia malayi infection in the ferret as an experimental animal model for occult filariasis in man. Initially a histopathologic study (88, submitted for publication) was made of this filarial infection in the ferret which confirmed the morphologic identity of the inflammatory response with the pathology reported in tropical (filarial) eosinophilia or the Meyers-Kouwenaar syndrome (89,90): the major clinical manifestations of occult infection in man. Further studies reported in this dissertation have considered principally the intravenous injection of microfilariae in ferrets as an experimental approach for the investigation of occult filariasis and have examined the immune mechanisms operative in the clearance of microfilariae from the circulation and the subsequent inflammatory responses to microfilariae characteristic of the tropical (filarial) eosinophilia syndrome.

Host Responses Following Mf Injection or L3 Infection

Comparison of host responses to microfilariae (mf) following i.v. injection of mf and following infection with third-stage larvae (L3) has demonstrated major similarities. These similarities included (1) the longevity of microfilaremia, (2) the association of circulating sheath-reactive antibody, predominantly IgG, with clearance of mf, (3) the development of immediate hypersensitivity to microfilarial

antigens and peripherial blood eosinophilia and (4) inflammatory cell infiltrates to mf which duplicated the focal lesions to mf described in tropical eosinophilia (91).

Differences in host responses following infection with third-stage larvae and following i.v. injection of microfilariae were also observed. There was a transient neutrophilia following the i.v. injection of mf perhaps caused by foreign materials or contaminants in the microfilarial inoculum. Ferrets infected with third-stage larvae usually demonstrated a higher degree of immediate hypersensitivity to microfilarial antigens, more liver lesions and a greater variability in eosinophilia and microfilaremia. These observed differences probably reflect the greater variability and complexity of host stimulation by experimental infections in contrast to the stimulation which follows i.v. injection of microfilariae. During infection, the host is exposed to several developmental stages of the parasite and to continued release of microfilariae produced by the female filariae, which precedes obvious microfilaremia and extends beyond the clearance of circulating microfilariae. Despite these differences, the similarities of the host responses to microfilariae, particularly the development of lesions typical of occult filariasis, support the use of intravenously injected microfilariae in the study of occult filariasis. In addition, the i.v. injection of microfilariae offers a much simplier and less variable model for analysis of immune mechanisms operative in the pathogenesis of occult filariasis.

Immune Mechanism of Microfilarial Clearance

Microfilarial clearance has been associated with serum antibodies to surface antigens of mf in several filarial infections of animals (40,47,48) and Brugia malayi in man (34,37). In the ferret the association between titer of IgG sheath-antibody and rapid clearance of mf following a second exposure to mf implicated an antibody-mediated immune clearance of microfilariae. The results of passive transfer studies demonstrated that the i.v. injection of immune sera, or the IgG-containing fraction of immune sera, rapidly cleared circulating microfilariae established either by i.v. injection of microfilariae or by infection with third-stage larvae and proved that clearance of B. malayi microfilariae could be serum-mediated. The recurrence of microfilaremia 2-3 weeks following microfilarial clearance by injected sera in ferrets infected with B. malayi third-stage larvae and the failure to clear circulating microfilariae of D. immitis further suggested that the effective antibodies were species-specific and probably recognized microfilarial-specific antigens; at least these antibodies did not damage the female filariae sufficiently to prevent the production of new microfilariae. Such stage-specific antibodies have been reported in other parasitic infections (92). Definitive proof that the humoral factor responsible for microfilarial clearance was IgG antibody with specificity for the microfilarial sheath was not obtained; however, the experimental results are consistent with the results of other investigators showing sheath-specific antibody is active in the clearance of circulating microfilariae in filarial infections (34,37,40,43,46,47).

Relationship of Microfilarial Clearance to Cell Adherence and Microfilarial Destruction

A number of experimental studies have indicated that antibodies with specificity for the surface of microfilariae act as opsonins, but do not directly destroy the microfilariae. Opsonized microfilariae are apparently trapped by cellular adherence reactions and are destroyed by a type of antibody-dependent cell-mediated cytotoxicity (93). The ability of antibody with specificity for the surface of microfilariae to promote cell adherence *in vitro* in filarial infections of experimental animal models and in lymphatic filariasis of man is well established (94-97). The class of immunoglobulin responsible for adherence varies, however, among animal models of filariasis. In hamsters, IgM antibody to the cuticle promotes cellular adherance to *D. viteae* microfilariae (95), while IgE in rats is implicated in cellular adherence to *Litomosoides carinii* and *D. viteae* microfilariae (94,96), whereas studies have suggested that IgG is responsible for osponizing microfilariae for cellular adherance in man (97).

The requirement of cells for the destruction of microfilariae has been confirmed in the *Brugia*-ferret model by studies with diffusion chambers (data not shown). Microfilariae placed in cell impermeable diffusion chambers (0.22 um pore size) and implanted subcutaneously into amicrofilaremic ferrets were, after 48 hours, agglutinated in a single large clump, but were still alive, whereas microfilariae placed in cell permeable chambers (5 um pore size) were surrounded by adherent leukocytes and were usually dead. Similar chambers implanted into control ferrets demonstrated no microfilarial agglutination and

little cell adherance. These results are identical to those reported by Weiss and Tanner (93) using golden hamsters and the filarial parasite Dipetalonema viteae. A detailed study of the mechanism and specific cells involved in the microfilaricidal activity has not been made in the ferret.

Receptors for both IgG and complement components have been identified on human neutrophils, eosinophils, and macrophages (98,99), and these cells have been implicated in the destruction of microfilariae. Although sheath-IgG antibody alone can promote cellular adherence to microfilariae, in the presence of complement an increase in cellular adherence has been observed (29-31). In vitro adherence studies of ferret peritoneal leukocytes to B. malayi mf (data not shown) confirmed an increased cellular adherence in the presence of IgG sheath-antibody and complement, but the role of complement in the clearance of microfilariae in vivo remains undetermined. Microfilariae opsonized in sera with high titers of sheath-IgM antibody fixed more complement in in vitro studies than did sera containing only sheath-IgG antibodies. However, sera with high titers of sheath-IgM antibody did not clear circulating microfilariae following passive transfer and microfilariae opsonized in these sera circulated at levels comparable to control microfilariae opsonized in normal sera, while sera with high titers of sheath-IgG antibody, which fixed less complement, were effective in both the opsonization and passive transfer of microfilarial clearance.

Histopathology of Occult Filariasis

As indicated previously, the inflammatory responses to microfilariae in infected ferrets are similar or identical to histopathologic responses reported in individuals with tropical (filarial) eosinophilia or the Meyers-Kouwenaar syndrome. These syndromes have been considered relatively uncommon events, probably dependent on immunologic hyperresponsiveness to microfilarial antigens (4), but features of the characteristic histopathology are also observed in other infections of man and animal (100). The allergic granulomas, leukocyte infiltrates and abscesses and particularly the presence of eosinophilic Splendore-Hoeppli deposits characteristic of occult filariasis are found in several bacterial, fungal and helminthic infections (100), but whether each of these diseases has a common pathogenesis is not known.

This dissertation has examined the histology and ultrastructure of the Splendore-Hoeppli (SH) material in the Meyers-Kouwenaar body in the ferret. The results have confirmed a structural similarity with the SH material observed in fungal (101) and other nematode infections (102) and implicated the granule proteins of the eosinophil and cell debris as major contributors to these deposits surrounding microfilariae. Comparison with descriptions of other examples of the Splendore-Hoeppli phenomenon suggests that the structural components vary, at least quantitatively, and that SH reactions may not have a common pathogenesis.

Several investigators have demonstrated that eosinophils are effective cytotoxic cells in immunity to certain helminths, including schistosomes (58,103,104), Trichinella (105), Onchocerca and

Dipetalonema (64,106). The ability of eosinophils to degranulate and release toxic proteins onto the surface of parasitic helminths has been suggested as a major functional role for these cells (106,107). The capablilty of eosinophils to phagocytize antigen-antibody complexes and to modulate immediate hypersensitivity responses have also been recognized (108-110). Unfortunately, the physiologic function of the eosinophil in the lesion of occult filariasis and the reasons for the excessive accumulation of SH material are not understood.

Blood Eosinophilia and Occult Filariasis

Ferrets injected intravenously with B. malayi microfilariae and normal or immune ferret sera developed a peripheral blood eosinophilia. The extent of blood eosinophilia was directly correlated with the number of surface liver lesions characteristic of occult filariasis at 1 and 3 weeks after microfilarial injection. Other investigators have reported a direct correlation between the degree of eosinophilia following microfilaricidal therapy and the extent of microfilaremia before therapy in bancroftian filariasis of man. It was suggested that this correlation represented a direct relationship between the degree of peripheral blood eosinophilia and the extent of inflammatory responses to microfilariae within the tissues (111).

The development of a primary and accelerated secondary peripheral blood eosinophilia has been shown in experimental animals to depend in many cases on a cell-mediated (T-lymphocyte) immune response (112,113). Antigen-specific T-lymphocytes, after contact with

homologous antigen, release a soluble substance (lymphokine) which stimulates bone marrow eosinophilopoesis (114). Many helminth infections induce some degree of eosinophilia and *in vitro* stimulation of lymphocytes from nematode-infected animals with homologous parasite antigen has been shown to induce an eosinophilopoetic factor (115). Whether the increase in blood eosinophils in ferrets injected with microfilariae and immune sera was the result of soluble eosinophilogenic substances present in the passively transfered sera, the reflection of active immune responses to the injected microfilariae or a combination of these events is not known at this time. Because the peripheral blood eosinophilia persisted for at least 3 weeks following i.v. injection of microfilariae, it is unlikely that this sustained eosinophilia is due solely to a release of mature bone marrow eosinophils in response to passively transferred eosinophil chemotactic factors.

Tissue Eosinophilia and Occult Filariasis

The development of eosinophilia and the eosinophil chemotactic stimuli which produce the focal inflammation to microfilariae are important aspects in the pathogenesis of occult infection. Both eosinophilia and eosinophil chemotaxis are now recognized to be complex in mediation and are incompletely understood as are the pathologic and physiologic roles of the eosinophils in inflammatory responses (116). Several chemotactic stimuli for the attraction of eosinophils have been identified in experimental inflammatory cell responses (116). Mast cell derived chemotactic factors for eosinophils (eosinophil chemotactic factor of anaphylaxis and

histamine), have probably been the most widely studied, and appear to be intimately associated with the lesions of occult filariasis in man (4,117) and in the ferret. In addition, mast cell products have been shown to enhance the cytotoxicity of eosinophils probably by increasing the number of membrane receptors for antibody or by enhancing the effectiveness of eosinophil peroxidase (116).

Sera collected from individuals with tropical pulmonary eosinophilia have been shown to contain IgE antibodies capable of sensitizing peripheral blood basophils from normal individuals to microfilarial antigens (118). Microfilariae which are secreting and/or excreting antigens into surrounding tissues or are releasing antigens following death could cause the degranulation of sensitized mast cells leading to the release of factors chemotactic for eosinophils. In addition, complement fixing antibody specific for microfilarial antigens could result in the degranulation of local mast cells through the biologic activity of C3a and C5a generated during the complement cascade (119). The degranulation of mast cells and basophils, in addition to the release of eosinophil chemotactic factors, have long been known to release histamine and bradykinin which are potent inducers of increased vascular permeability. Because the migration of eosinophils into tissues occurs by diapedesis, factors which increase vascular permeability also facilitate the accumulation of eosinophils (120).

The development of tissue eosinophilia in response to a parasitic extract, Ascaris suum, has been studied in sensitized guinea pigs and was shown to be biphasic (121). The initial phase of tissue eosinophilia occurred at 6 hours and was associated with the

degranulation of mast cells, while the second phase of tissue eosinophilia occurred at 24 hours and was correlated with the presence of a heat-labile factor compatible with the lymphocyte derived eosinophil chemotactic factors: eosinophil chemotactic factor precursor (ECFp) and eosinophil stimulation promoter (ESP). Eosinophil chemotactic factor precursor has been shown to be produced from antigen-specific lymphocytes upon challenge with homologous antigen, but requires modification by exposure to homologous antigen-antibody complexes before it becomes chemotactic for eosinophils (122). Eosinophil stimulation promoter (ESP) is similar to ECFp, is produced by lymphocytes following mitogen- or antigen-activation, but does not require modification by immune complexes (123,124).

Ferrets infected with Trichinella spiralis exhibited no detectable immediate hypersensitivity and little cross-reactive humoral responses to microfilarial antigens. However, 15 of 15 ferrets infected with T. spiralis and exposed to an intravenous injection of B. malayi microfilariae, develped typical lesions of occult filariasis. Wedge liver biopsy at 1 and 2 days following the intravenous injection of microfilarae did not demonstrate a marked accumulation of eosinophils and no deposition of material characteristic of Splendore-Hoeppli substance. However, at 4-7 days, typical accumulation of enormous numbers of eosinophils along with the deposition of characteristic Splendore-Hoeppli material were seen. Taken together, these results are compatible with the activity of a lymphocyte derived eosinophil chemotactic factor(s), but do not exclude other possible mechanisms. Although the asthma-like clinical

characteristics of tropical pulmonary eosinophilia are usually associated with immediate hypersensitivity to microfilarial antigens, typical lesions of occult filariasis are present in several organs, probably often clinically inapparent, and cell-mediated immune responses to microfilarial antigens may be significant in their pathogenesis (67,125,126). Experiments are currently in progress to identify cell-mediated immune responses in the ferret which could contribute to the pathology of occult infection, in particular the eosinophil response.

Rapid Microfilarial Clearance and Histopathology

The rapid clearance of i.v. injected microfilariae in normal ferrets, following passive transfer of sera with antibody to the microfilarial sheath, did not result in detectable inflammatory responses to microfilariae at 1 or 3 weeks following microfilarial clearance. Histologic examination of tissue obtained 24 hours after rapid clearance of microfilariae revealed small foci of mixed inflammatory cells in liver, spleen and lungs which apparently resolved rapidly. These results demonstrated that microfilariae can be cleared and destroyed by host immune responses with little obvious pathology. In contrast, the majority of ferrets injected only with microfilariae developed lesions characteristic of occult infection within 3 weeks and showed only a marginal reduction in numbers of circulating microfilariae. Ferrets infected with Trichinella and subsequently injected with microfilariae consistently developed lesions typical of occult filariasis within 1 week and also did not show marked reduction in microfilaremia. Intravenous injection of

immune sera effective in the rapid clearance of microfilariae in ferrets infected with T. spiralis and injected with microfilariae resulted in a few surface liver lesions at 4 days and complete resolution of these lesions by 17 days following microfilarial clearance; control ferrets infected with T. spiralis and injected with only microfilariae showed numerous lesions at both time periods. These observations suggest the possibility that antibody to microfilariae which induce rapid microfilarial clearance, may partially block or bypass immune responses which induce the pathology of occult infection. Critical studies have not been carried out in the ferret to test this hypothesis. Blocking antibody for in vitro IgE-dependent histamine release from basophils by microfilarial antigens has been demonstrated in lymphatic filariasis of man (127); although actual modulation of hypersensitivity by antibody in vivo has not been proven. The modulation of host responses by immunoregulatory mechanisms probably determine, to a major degree, the pathology of filarial infection and the ferret may represent a potential experimental animal to examine this important aspect of filarial infection.

CONCLUSION

This investigation has demonstrated a marked similarity of inflammatory responses to microfilariae (mf) following infection with *B. malayi* third-stage larvae in the ferret with the inflammation reported in tropical (filarial) eosinophilia or the Meyers-Kouwenaar syndrome of man. The intravenous (i.v.) injection of mf was shown to offer a convenient method to study immunologic mechanisms active in microfilarial clearance and the pathogenesis of occult filariasis. Passive transfer of immune sera and sera fractions and the *in vitro* opsonization of mf proved that microfilarial clearance was serum-mediated and suggested that clearance was mediated by sheath-specific IgG antibodies which were also species-specific.

Ultrastructural and histochemical studies of the typical lesion of occult filariasis indicated that degranulating eosinophils contributed to the formation of the Meyers-Kouwenaar (MK) body and that resolution of the MK body was accomplished within granulomas. The typical lesions of occult filariasis were associated with immediate hypersensitivity to microfilarial antigens and passive transfer of sera with high titers of microfilarial-specific reaginic antibody was shown to induce accelerated lesion development. Rapid clearance of mf did not lead to lesion formation. These observations indicate that microfilarial clearance is insufficient to induce the development of lesions and that immediate hypersensitivity is probably an immune mechanism active in the pathogenesis of occult infection.

Ferrets infected with Trichinella and injected with mf exhibited typical lesions of occult filariasis without detectable immediate hypersensitivity to microfilarial antigens and microfilariae rapidly cleared by passive transfer of immune sera in Trichinella infected ferrets resulted in fewer lesions as compared to control animals. These results suggest that although immediate hypersensitivity to microfilarial antigens may represent an immune mechanism active in the pathogenesis of occult filariasis the typical lesion may be induced by other immunologic mechanisms and that passive transfer of sera effective in the rapid elimination of mf may partially block or bypass immune responses which induce the pathology of occult infection.

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BIOGRAPHICAL SKETCH

James P. Thompson was born June 1, 1954, in Greenville, Pennsylvania. He attended Purdue University from 1972-1976 and earned the B.S. degree in biology. During his education at Purdue, he participated in collegiate athletics as a polevaulter, lettered all four of his undergraduate years, set the University indoor polevault record, and was team captain his senior year. Following graduation from Purdue, he entered graduate school in the Department of Immunology and Medical Microbiology, College of Medicine, University of Florida. After 1 year of graduate study, he entered the College of Veterinary Medicine with the intent of continuing his research training. On November 15, 1980, he married Joan A. Chadwell. He graduated from the College of Veterinary Medicine with honors in June 1981 and immediately returned as a full-time graduate student to continue research training under the direction of Drs. Richard and Catherine Crandall. On February 28, 1983, Joan gave birth to their first child, James Paul; their second child is expected in June 1984. Following the completion of his dissertation, he intends to continue investigative research in immunology and to persue residency training in Veterinary Internal Medicine in the hopes of making significant contributions to the knowledge of medicine.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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