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IMMUNE RESPONSES AND CHRONIC LYMPHEDEMA IN EXPERIMENTAL FILARIASIS

R. Crandall, C. Crandall, T. Doyle, J. Nayar

Departments of Immunology and Medical Microbiology (RC,TD), and Pathology (CC), University of Florida, Gainesville, Florida, Florida Medical Entomology Laboratory (JN), Vero Beach, Florida, USA

ABSTRACT

Chronic lymphedema is a clinically important manifestation of lymphatic filariasis but the factors which govern the development and progression of lymphedema remain unclear. Because immune responses are major determinants of disease expression in filariasis, we compared immune responses in ferrets reinfected with B. malayi *in which disease expression varied from virtually no overt disease to severe chronic lymphedema of the infected limbs. No immune correlates specific for development of chronic lymphedema were identified by antigen recognition profiles (Western-blots), magnitude of the antibody (IgG) responses (ELISA) or blastogenic responses of lymphocytes to filarial extracts. Prausnitz-Kustner (PK) tests for filarial specific IgE indicated that the initial period of a severe lymphedema, which became chronic, often was associated with relatively high IgE titers. The results suggest that a high level of immediate hypersensitivity to filarial antigens is a significant factor in initiating persistent lymphedema on reinfections of partially resistant hosts. Histologic study of persistent edema of up to 2 years duration demonstrated dermal changes consistent with chronic lymphedema, but not the dermal proliferation characteristic of elephantiasis.*

Lymphatic filariasis presents a spectrum of clinical manifestations thought to be immunologically mediated (1). Of these manifestations chronic lymphostatic disease, which occurs in a minority of infected individuals, is of major importance (2). The host-filarial parasite interactions inducing lymphedema and the factors responsible for progression to chronic disease remain poorly defined and knowledge of these factors is potentially of clinical significance (2). To investigate the role of immune responses in the development of chronic lymphedema a longitudinal, comparative examination of immune responses was made in ferrets infected with *B. malayi*. This experimental host develops lymphatic dysfunction comparable to that reported in human infection (3). In two previous infection studies in ferrets, the development of lymphostatic disease and associated histopathology has been described (4,5). This study describes the immune responses assayed in these earlier studies and examines their relationship to the development and persistence of lymphedema. In addition, a limited histologic study was done to characterize further dermal changes in chronic lymphedema of infected ferrets.

MATERIALS AND METHODS

Animals and Infections

Ferrets were males, 6 months to 1 year old, purchased from Marshall Research Animals, North Rose, NY. The experimental groups

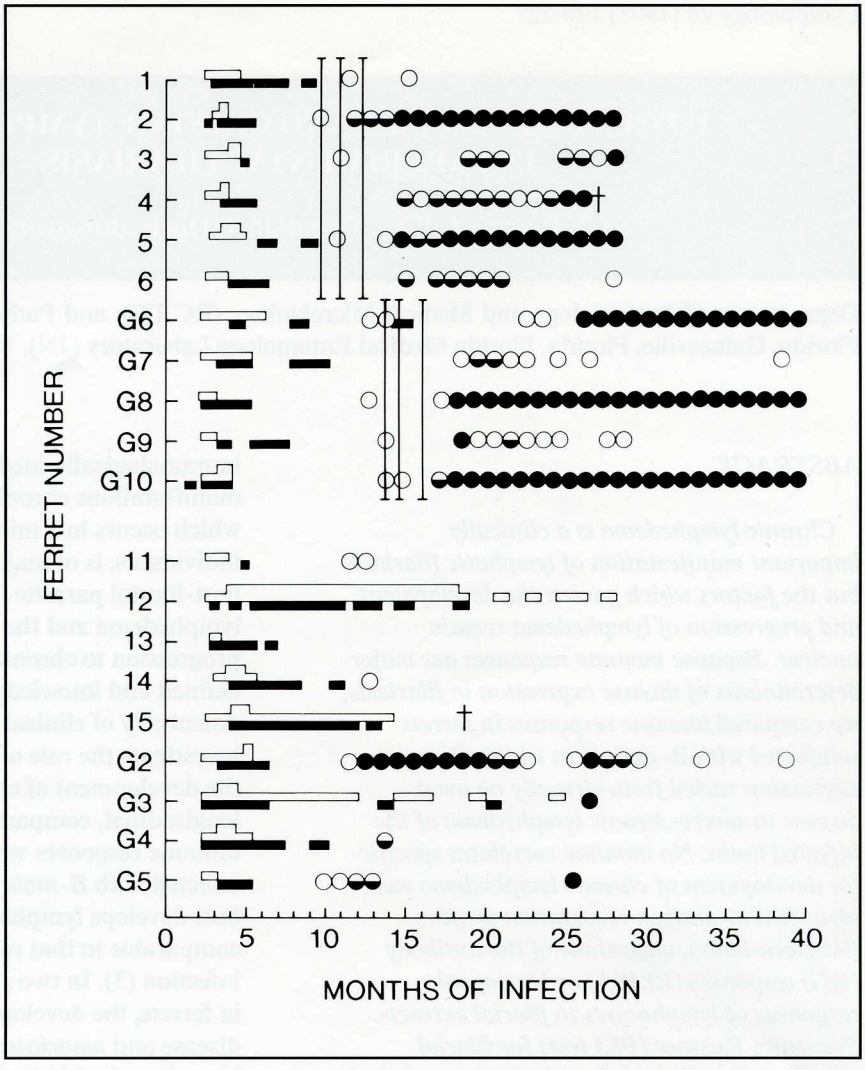

Fig. 1. Lymphedema, microfilaremia and eosinophilia in ferrets following single inoculation and reinoculations of infective larvae of Brugia malayi. Each ferret was inoculated initially in both hindpaws; ferrets 1-6 reinoculated in right hindpaw and G6-G10 reinoculated in right inguinal area. Circles=lymphedema: open circle=probably edematous change; half closed circle=definite enlargement of paw or paw and lower limb; closed circle=conspicuous edema (>50% volume increase in paw and limb). Open bar=microfilaremia (mf) (wide bars=>1000 mf/ml, narrow bars=<1000 mf/ml); closed bars=eosinophilia ((>2000/µl); Vertical lines=reinoculations; Crosses=ferret death.

have been described previously (4). Ferrets 1-6 and 11-15 *(see Fig. 1)* were inoculated in the hindpaws with 75 infective larvae (L3) and at 10 months ferrets 1-6 were reinfected by 3 inoculations in the right hindpaw with 30 L3 at 1 month intervals. Infected ferrets were observed for approximately 2 years. In a second study, ferrets G2 to G10 were inoculated with 75 L3 and 5 of these ferrets (G6-G10) were reinfected by 3 inoculations of 50 L3 in the right inguinal area *(see Fig. 1)*; the ferrets were observed over a 3 year period. Histopathology of this group with attention to the lymphatic and vascular changes has been reported (5). Procedures for infecting ferrets with *B. malayi*, measuring microfilaremia, peripheral blood leukocyte responses and lymphedema have been described (4,6,7).

Antigens

Adults and microfilariae (mf) of *B. malayi*

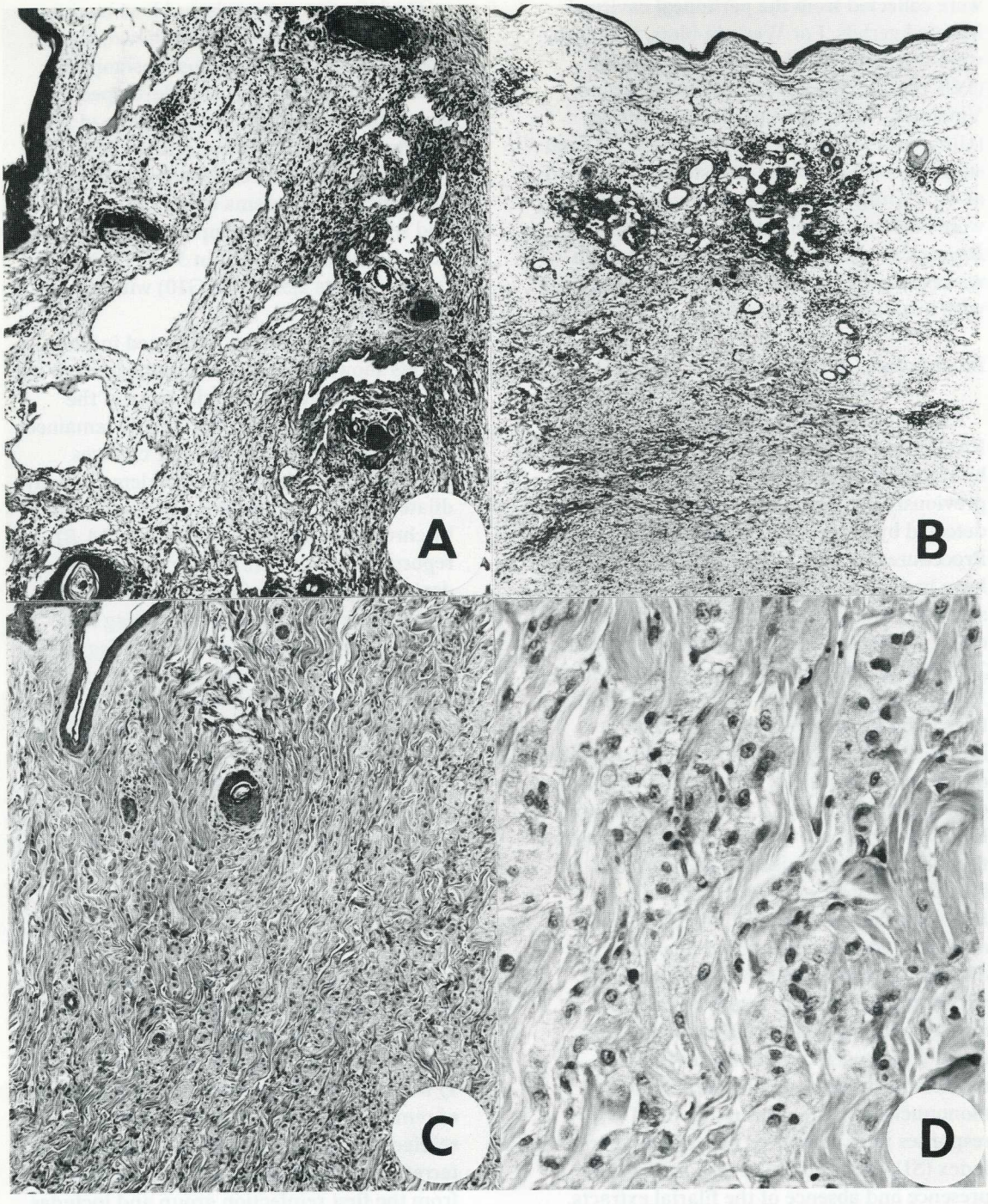

Fig. 2. Dermal histopathology in chronic lymphedema of ferrets infected with B. malayi. A) Dorsal skin of edematous paw showing dilated lymphatics in superficial dermis and B) lymphatics surrounded by chronic inflammatory cells. C,D) Thickened inguinal skin at site of edema showing infiltration with vacuolated histiocytes and multinuclear cells. Original magnification A,x40; B,x25; C,x25; D,x100.

were collected from the peritoneal cavity of infected gerbils. For Western-blots, helminths were washed with distilled water, ground (Pellet Pestle, Knotes Scientific Glassware, Vineland, NJ) or disrupted by sonication (Ultrasonics Model W-10, Plainview, NJ) and suspensions lyophilized and stored desiccated at 4°C. Antigens for other immunologic assays were buffered saline extracts of mf and adult *Brugia* disrupted in a pressure cell. Protein was estimated by a Bio Rad Protein Assay with ovalbumin as a standard.

Immunologic Assays

Electrophoresis on gradient gels (SDS-PAGE) and immunoblotting were carried out by standard procedures (8) as described previously (9). Immunoglobulin-binding was detected by peroxidase-labeled Protein A (9). Procedures for skin tests, PK tests, and ELISA have been described (6,9). Briefly, for direct skin tests intradermal injections of filarial extracts were made at 10 fold dilutions from 2µg to 0.002µg of protein per site and read at 15 min for development of erythema. In PK tests uninfected ferrets were injected intradermally with dilutions of 1:200 to 1:3200 of test sera followed in 72 hrs by the injection of 0.2µg of filarial extract into the same site. Evans blue dye was injected intravenously at the time of extract injection and dermal blueing reactions were read at 15 min following the dye-injection. In ELISA, antibody (IgG) was measured in a single dilution, 1:1000, of test sera and compared with standard positive serum by optical density readings (OD at 405nm). Measurement of blastogenic responses to filarial extracts in cultures of peripheral blood mononuclear cells has been described (9); responses were compared by a stimulation index (SI), the ratio of the responses in the presence and absence of the filarial extracts.

RESULTS

Responses to Infection

The infection protocols and host responses are summarized in *Fig. 1.* As shown, ferrets, post-microfilaremia and post-eosinophilia, after a single infection often developed transient, usually mild lymphedema of the infected paw and limb but seldom persistent lymphedema. After reinfections many ferrets developed lymphedema of varying duration and severity; chronic lymphedema of over 1 year duration developed in 5 of 11 reinfected ferrets (ferrets 2,5,G6,G8,G10) with no sign of remission.

Dermal changes were examined in those ferrets showing persistent lymphedema of up to 2 years duration. Lymphedema of the infected paw and lower leg usually remained soft and spongy with histologic changes consistent with chronic lymphedema namely dilated dermal lymphatics, often surrounded by chronic inflammatory cells *(Fig. 1A,B).* As reported previously (5), the conspicuous dermal hyperplasia of elephantiasis was not present; however, dermal thickening and hardening were noted in the upper leg and inguinal areas in two edematous ferrets. Histologic examination of the thickened dermal areas showed collagen deposition and infiltrates consisting mostly of mononuclear and multinucleate cells *(Fig. 2A,B)*; the infiltrating cells often had foamy cytoplasm and stained intensely for lipid with Oil Red O *(Fig. 2C,D).*

Immune Responses

Immunoblots and ELISA were used to compare antigen recognition profiles and the magnitude of the antibody (IgG) responses *(Fig. 3).* Sera from the reinfected ferrets *(Fig. 1)* were assayed by immunoblotting at the time of reinfection, at 10-12 months after the first reinfection and again at 36 months in the G ferrets. *Fig. 3B-D* shows selected immunoblots from the first reinfection group and includes ferrets with essentially no overt edema (ferret 1), with chronic lymphedema (ferrets 2 and 5) and with a single episode of edema (ferret 6). As shown, the antibody binding patterns on

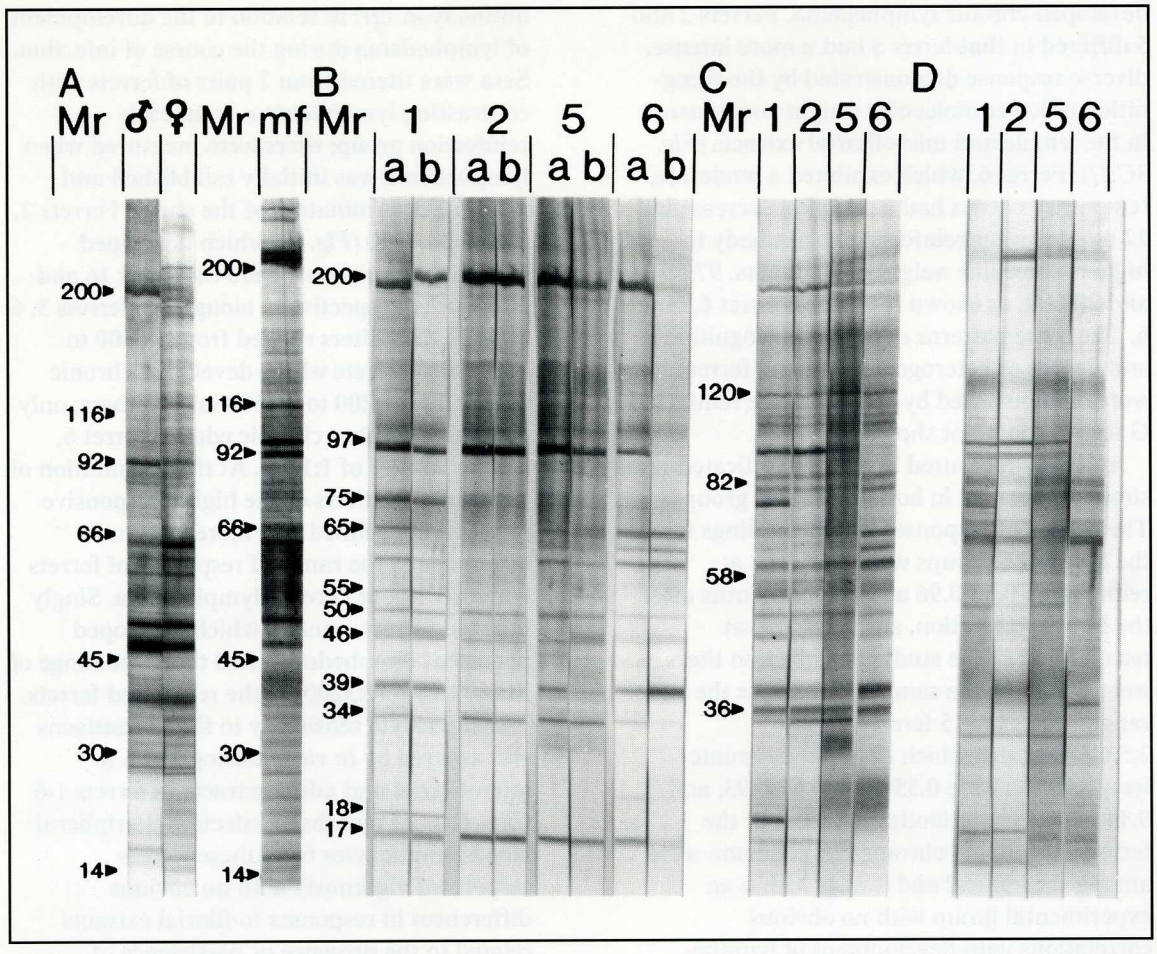

Fig. 3. Immunoblots with sera from reinfected ferrets with different degrees of lymphedema. A. India Ink stain of western blots from SDS-PAGE of female, male and microfilarial extracts of Brugia used for immunostaining. Mr=position of molecular weight markers in kD. B. Immunoblots of male extract with sera from ferrets, 1,2,5,6 (Fig. 1) at (a) reinfection and (b) at 12 months after initial reinfection. Mr=relative molecular weights of major antibody binding bands. C. Immunoblots of same sera on female extract at 12 months. Mr=molecular weights of some additional components recognized in female extract. D. Immunoblots as in C on microfilarial extract.

male and female extracts were similar but with several additional bands on the more complex female extract *(Fig. 3C)* and a number of differences in pattern between the microfilarial *(Fig. 3D)* and adult extracts. Preinfection sera were assayed and light, variable Ig-binding was observed in a few sera at 90, 60-65, 60 and 34 kd with the female extract (not shown).

Comparison of antigen recognition profiles of the 5 ferrets which developed chronic lymphedema with other ferrets in the experimental groups identified no consistent differences associated with chronic lymphedema. There was, however, heterogeneity of Ig-binding among ferrets indicating differences in both specificity and magnitude of the antibody response. As illustrated in *Fig. 3B*, sera from ferret 1, which developed essentially no lymphedema, demonstrated Ig-binding at 75, 55 and 18 kd which was less evident in ferrets 2 and 5, both of which

developed chronic lymphedema. Ferrets 2 and 5 differed in that ferret 5 had a more intense, diverse response demonstrated by the recognition of lower molecular weight components in the female and microfilarial extracts *(Fig. 3C,D)*. Ferret 6, which exhibited a moderate, temporary edema had a selective decrease by 12 months after reinfection in antibody to higher molecular weight components, 97 kd and 200 kd, as shown in *Fig. 3B*, ferret 6, lane b. The same patterns of antigen recognition and degree of heterogeneity among ferrets were demonstrated by sera from the reinfected G ferrets (data not shown).

Antibody measured by ELISA indicated similar responses in both reinfected groups. The ranges of responses by OD readings for the combined groups were 0.50-1.08 at reinfection, 0.58-0.96 at 10 to 12 months after the initial reinfection, and 0.44-0.94 at termination of the study. Preinfection titers were <0.08. At the same time periods the ranges of just the 5 ferrets (ferrets 2,5,G6,G8,G10) which developed chronic lymphedema were 0.55-1.08, 0.61-0.93, and 0.44-0.94. The antibody responses of the ferrets developing chronic lymphedema were among the highest and lowest within an experimental group with no obvious correlations with development of lymphedema. Sera from the singly infected G ferrets (G2-G5) were assayed also at the same time periods and gave responses equivalent to the reinfected G ferrets except for an apparent decline in antibody by 35 months in most ferrets; the response ranges were 0.53-1.04, 0.34-0.91 and 0.26-0.61.

Ferrets were skin-tested for immediate hypersensitivity at necropsy, or at 35 months (G ferrets). All infected ferrets were sensitive to 0.2µg of adult and microfilarial extracts; uninfected ferrets were not reactive. There were no consistent differences in sensitivity to adult and microfilarial extracts and no obvious correlation of chronic lymphedema with cutaneous sensitivity which ranged from 0.02µg to 0.002µg in reinfected ferrets. Prausnitz-Kustner tests were used to measure antibody in IgE in relation to the development of lymphedema during the course of infection. Sera were titered from 2 pairs of ferrets with contrasting lymphedemas from each reinfection group; titers were measured when lymphedema was initially established and again near termination of the study. Ferrets 2, 5 and G8, G10 *(Fig. 1)*, which developed chronic lymphedema, were titered at 16 and 20 months, respectively, along with ferrets 3, 6 and G7, G9. Titers ranged from 1:1600 to >1:3200 in ferrets which developed chronic edema, and 1:200 to 1:1600 in the others; only one ferret without chronic edema, ferret 6, reached a titer of 1:1600. At the termination of the study, the titers of the highly responsive ferrets had declined and there was no difference in the range of responses of ferrets with or without chronic lymphedema. Singly infected ferrets, none of which developed persistent lymphedema, had the same range of titers, 1:200->1:3200, as the reinfected ferrets.

Lymphocyte sensitivity to filarial antigens was assayed by *in vitro* blastogenesis to microfilarial and adult extracts in ferrets 1-6 *(Fig. 1)* at 24 months of infection. Peripheral blood lymphocytes from these ferrets responded vigorously with no obvious differences in responses to filarial extracts related to the presence or persistence of lymphedema. The mean stimulation indices (SI) for the 6 ferrets were 20.0 (SE 2.5), 22.1 (SE 6.5) and 74.8 (SE 8.5) for microfilarial extract, adult extract and the mitogen, Concanavalin A, respectively; in two uninfected control ferrets the indices were 1.4, 1.3 and 67.0, respectively. The SI was calculated for a single optimum concentration of extract (5µg/ml) and mitogen (1µg/ml).

DISCUSSION

The objective of this study was to identify immune correlates for the development of chronic lymphedema. The responses to reinfection in ferrets corresponded to the clinical expressions generally associated with a high immune responsiveness which include

chronic lymphedema as well as a lack of obvious disease or infection at the time of examination, the "endemic normals" (1). Although the nature and magnitude of immune responses are considered to determine clinical presentation, cross-sectional studies in endemic areas have not immunologically differentiated chronic lymphedema from endemic normals by antigen recognition profiles or isotypes of the antibody responses (10,11). Similarly, this study demonstrated a high immune responsiveness in reinfected ferrets but did not identify responses specifically associated with chronic lymphedema, as compared to transient edemas or no edema, except for a tendency of a severe, persistent lymphedema to be associated initially with higher PK titers.

The PK tests for filarial specific IgE suggest that a relatively high immediate hypersensitivity may be a factor in initiating persistent lymphedema after reinfections. Immediate hypersensitivity has been implicated in the induction of filarial lymphedema (12) and high protein edema, in itself, prolonged by compromised lymphatic function, could promote fibrosis and other tissue changes leading to chronic disease (13) without a continuing association with hypersensitivity. The limited results in this study offer no direct proof of a role of immediate hypersensitivity in the development of lymphedema. Subsequent, unpublished studies, however, have demonstrated that ferrets after initial infection develop partial resistance to lymphatic stages of *Brugia* and to microfilaremia, and it is only the resistant ferrets with the higher levels of immediate hypersensitivity to filarial antigens that frequently develop overt lymphedema following a challenge infection.

Although development of lymphedema in filariasis does not necessarily depend upon immune responses, it has been suggested that repeated, hypersensitivity-mediated inflammatory episodes can result in permanent lymphatic damage and chronic lymphostasis (14). In the ferret, an immunologically hyperresponsive host (6), adenolymphangitis and impaired lymphatic function of the infected limbs are induced (15,16) with the development of transient edemas, but rarely persistent edemas, following initial infection. As demonstrated in this study, chronic lymphedema follows reinfections in postmicrofilaremic ferrets that have retained relatively high levels of immediate hypersensitivity to filarial antigens and presumably have acquired partial resistance to reinfection. These results suggest that an immediate hypersensitivity presents a significant risk for the development of sustained lymphedema following reinfections in partially resistant hosts. More detailed study may identify patterns of response to specific antigens useful for evaluating the risk of chronic lymphedema with repeated exposures to filarial infection.

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Catherine A. Crandall, Ph.D.
Department of Pathology &
Laboratory Medicine
University of Florida College of Medicine
P.O. Box 100275
Gainesville, FL 32610-0275 USA