ASSOCIATION OF ELEVATED LYMPH NODE CELL RELEASE OF HISTAMINE AND TUMOR NECROSIS FACTOR WITH GENETIC PREDISPOSITION TO LIMB EDEMA FORMATION IN DOGS INFECTED WITH BRUGIA PAHANGI

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Abstract. Brugia pahangi infection in the canine rear limb results in marked lymphatic duct and popliteal lymph node pathologic changes. Limb edema is variably associated with infection and does not correlate well with duct or node lesions. To understand the mechanisms of limb edema, lymph node cells were collected by sequential biopsy following infection and examined for production of inflammatory mediators. Lymph node cells from a litter of dogs selectively bred with a high incidence of edema formation (82%) demonstrated spontaneously released histamine and prostaglandin E2 levels higher than those of closely related nondenema-forming dogs (0–20%) and/or control dogs. These edema-forming dogs also showed elevated release of tumor necrosis factor-α when cells were cultured with Brugia antigen. Toluidine blue staining of infected lymph node sections revealed that the edema-forming dogs had higher numbers of mast cells than infected lymph nodes of nondenema-forming dogs.

Our earlier studies of dogs infected in lymph ducts afferent to the popliteal lymph node and in the capsule of the node itself with Brugia pahangi showed marked changes in lymphatic duct structure that included dilation and rechannelization, as well as variable loss of function as indicated by dye leakage from ducts. Whereas loss of function indicated by dye leakage correlated well with edema formation, the structural changes, no matter how dramatic, did not correlate with coincident or subsequent edema manifestation.

This is similar to observations made in human lymphatic filariasis cases. These observations support the hypothesis that edema formation in lymphatic filariasis involves complex host-parasite interactions in addition to or exclusive of dilation and deformation of lymphatic ducts, which have been proposed to be associated with the mechanical presence and physical activity of adult worms in ducts. The host-parasite interactions most often cited and studied in lymphatic filariasis are those dependent upon an active immune response involving antigen-specific and antigen-nonspecific components.

In our description of the interaction of the lymphatic filarial parasite, B. pahangi, with its natural host, the dog, we have reported acute and chronic pathologic lesions, pathogenesis, and immunologic responses that are nearly identical with those reported in human lymphatic filariasis. In addition, we have reported on a strong familial association between amicrofilaraemia and edema formation in its acute and chronic manifestation.

Chronic lymphedema occurs in varying degrees and has been traditionally selected as the clinical sign to be used for correlations with host responses in studies seeking to elucidate mechanisms of pathogenesis. The importance of acute episodes of transient edema and local lymphadenitis as risk factors for chronic lymphedema has been clearly demonstrated in longitudinal studies of humans. These retrospective studies involving historical data of acute episodes are difficult to execute and there are few available in the literature; yet, correlations of host responses to episodes of acute, transient edema are valuable in elucidating mechanisms of pathogenesis in lymphatic filariasis.

No strong correlations between lymphatic disease and the various types of parasite antigen-specific immune responses, including immunoglobulin subclass quantitation and T cell cytokine measurement, have been identified after nearly 20 years of searching for this likely association. This lack of correlation is even more remarkable given that a familial tendency to develop chronic edema has been recognized since 1981. Given the apparent complexity of factors involved in host-parasite interactions leading to manifestation of edema as suggested above, it is not surprising in the absence of associations between immune responses and clinical disease, that the genetic tendency toward manifestation of edema in human lymphatic filariasis is not readily understood.

We observed the manifestation of edema at onset of patency during primary infection with Brugia in 82% of the offspring of a particular brother × sister mating in beagle dogs. This led us to attempt to quantitate selected inflammatory mediators locally produced during infection of these dogs. Comparisons were made between edema-forming dogs and dogs that were closely related but not manifesting a high frequency of edema.

MATERIALS AND METHODS

Animals and infection regimen. Fifteen beagle dogs from four litters (E = edema-forming; NEa = nondenema-forming litter #1; NEb = nondenema-forming litter #2; and UC = uninfected control) were raised and maintained in closed housing, without exposure to helminth parasites. Phenotypic status (E or NE) was determined by the risk for development of acute limb edema formation at the onset of patency after infection, as judged by previous breeding and infection experiments. At the time puppies were born all mothers had patent Brugia infections. Brugia pahangi infective larvae (L3) were obtained as previously described from infected jirds. All infections were performed by subcutaneous injection of L3 in the dorsum of the left rear paw. All the dogs in litters E, NEa, and NEb received 20 L3 at the age of 12 weeks, whereas the UC dogs remained uninfected.

Surgical biopsies. Surgical biopsies from infected and un-
infected lymph nodes were taken at different time-points postinfection as described previously.\textsuperscript{17} This surgical biopsy does not reduce lymph flow through the lymph node as demonstrated by xeroradiography.\textsuperscript{18} Biopsies of the left popliteal lymph node (infected) and the right popliteal lymph node (uninfected) were taken at 5.5–6 (immediately prior to edema formation [S1]) and 8.5–9 weeks (just after onset of edema [S2]) post infection from all infected dogs. Biopsies from uninfected dogs UC1 and UC2 were taken at the age of 25 and 29 weeks. Lymph node biopsies were placed in RPMI 1640 medium supplemented with 100 U/ml of penicillin and 100 μg/ml of streptomycin (RPMI-PS) (Gibco, Grand Island, NY) at 4°C. A small piece of each biopsy was fixed in 10% neutral-buffered formalin, embedded in paraffin, and used for histochemical and immunohistochemical procedures. Paraffin-embedded sections were stained with hematoxylin and eosin, and with 0.5% toluidine blue for quantitation of mast cells present in the lymph nodes.

**Antigens.** The *Brugia pahangi* antigen (BpA) preparation was a saline soluble extract of male and female adult worms recovered from the peritoneal cavity of experimentally infected jirds as described previously.\textsuperscript{1}

**Preparation of cell suspensions.** Portions of lymph node biopsies were teased and prepared as single cell suspensions. Lymph node cell suspensions from infected and uninfected lymph nodes were cultured in flat-bottom tissue culture plates (Costar, Cambridge, MA) at $5 \times 10^5$ cells/well in a final volume of 200 μl of RPMI-PS supplemented with 2 mM L-glutamine, 25 mM HEPES, and 0.2% bovine serum albumin. Six replicate cultures were stimulated with BpA (0.1 μg/ml) or left unstimulated and incubated for 24 hr at 37°C in a 7% CO₂ humified incubator. After 24 hr, supernatants of the six replicate cultures were harvested as two pools of three wells each to assess experimental variation. Additional media was added to the wells and cultures were continued for the production of immunoglobulins (results previously published)\textsuperscript{17} and thus were not available for quantitation of intracellular inflammatory mediators. Samples were frozen at -70°C.

**In vitro assays.** Histamine (HA) levels were measured in 24-hr lymph node cell supernatants by competitive inhibition ELISA (Miles Inc., Westhaven, CT). Results are expressed in nanomoles/liter in relation to histamine standards. There were not enough cells per biopsy to allow a separate series of cultures to be set up so that supernatants and pellets could be harvested for histamine, thus allowing us to only measure absolute histamine concentrations in the supernatants and not percent histamine released from the cells. Prostaglandin E2 (PGE-2) levels were measured using a radioimmunoassay kit also based upon competitive inhibition of binding (New England Nuclear, Wilmington, DE) following extraction of sample with ethyl acetate and methanol and reported as pg/ml using PGE-2 as a standard. Bioactive tumor necrosis factor-α (TNF-α) was assayed by in vitro bioassay based on killing of L929 mouse fibroblast cells.\textsuperscript{19} Briefly, L929 cells were seeded at a concentration of $2.5 \times 10^4$ cells/well in a 96-well, flat-bottom tissue culture plate (Costar) and incubated at 37°C in 7% CO₂. After 24 hr, the supernatant was removed and 100 μl of samples containing TNF-α were added along with 100 μl of actinomycin D at a concentration of 2 μg/ml and the plates incubated for another 16 hr. Samples were poured off the cells and blotted dry followed by addition of 100 μl of 0.2% crystal violet and incubated for 15 min. The plates were washed five times with water and remaining adherent cells were lysed with 6 M acetic acid. Absorbance was read at 570 nm. Recombinant human TNF-α (Genzyme, Inc., Cambridge, MA) was used as a standard with results being reported as units/ml. One unit of TNF was defined as the amount of TNF required to kill 50% of the cells in a well.

**Blastogenesis assay.** Lymph node cells from infected and uninfected limbs were cultured in flat-bottom tissue culture plates (Costar) at $5 \times 10^5$ cells/well in a final volume of 200 μl of RPMI-PS supplemented with 2 mM L-glutamine, 25 mM HEPES, and 10% heat-inactivated normal dog serum. Cells were unstimulated or stimulated with BpA (10 μg/ml) or the mucoprotein form of phytohemagglutinin (5 μg/ml) (Sigma, St. Louis, MO). Triplicate cultures were incubated for three and five days at 37°C in a 7% CO₂ humidified incubator before pulsing with 1 μCl/l of $^{3}H$-thymidine (New England Nuclear) for 24 hr. After incubation, samples were harvested on a PHD® cell harvester (Cambridge Technology, Watertown, MA) and counted using β-emission liquid scintillation spectroscopy. Results are expressed as mean counts per minute (cpm). The higher value of the four versus six-day cultures was reported to represent response to BpA.

**Immunohistochemical staining.** Four micron-thick paraffin-embedded lymph node sections were first deparaffinized and rehydrated to H₂O. The slides were then incubated for 30 min in 0.3% H₂O₂ to block endogenous peroxidase reactivity. Following a wash step with H₂O, then phosphate-buffered saline (PBS), and a 20-min incubation in 5% normal goat serum, the slides were incubated with polyclonal anti-human TNF-α (Genzyme, Inc.) for 30 min at room temperature. The slides were then washed three times in PBS and incubated for 20 min with biotinylated goat anti-rabbit immunoglobulin (supsensitive kit; Biogenex, San Ramon, CA). After another three washes, streptavidin-conjugated peroxidase (Biogenex) was added to the slides and incubated another 20 min at room temperature. After a final series of washes the slides were developed with 3-amoeno-9-ethyl-carbazole in N, N-dimethyl formamide in acetate buffer, pH 5.2. The color reaction was stopped by washing the slides with H₂O. To determine specificity of the antibody to TNF-α, we added human recombinant TNF-α to the primary antibody immediately prior to incubation of the antibody with the lymph node sections.

**Statistical analysis.** Significance of differences between litters for the various parameters tested was evaluated using the Student’s t-test assuming unequal variances. Any $P$ values less than 0.05 were considered significant.

**RESULTS**

**Clinical manifestations.** Figure 1 shows the familial relationships of selectively bred siblings yielding offspring with high or low risk for acute edema formation at the onset of patency 7–8 weeks post infection. Dogs E2, E3, and E4 from one litter and dog NEb5 were the only dogs included in this study that showed limb edema. Dogs E2, E3, and E4
were siblings of individuals from two other litters by the same parents, and the same high incidence of limb edema and amicrofilaremic infections was described in detail for one of these litters.17 Litter NEa was the result of a brother × sister mating of dogs that consistently demonstrated high microfilariaemia and were asymptomatic, this mating resulted in four dogs that did not manifest limb edema even though patent infections were established. Dogs in litter NEb were from another brother × sister mating, both the sire and the dam exhibited chronic limb edema. Only one dog, NEb5, from this litter of five dogs developed limb edema. All dogs from litter E and their parents, R1 and R2, showed pronounced swelling of the infected lymph node at four weeks post-infection. In all other infected dogs, pronounced swelling of the lymph node was not observed before seven weeks post-infection. Patency was judged by the observation of microfilaria in cell preparations from the lymph node that drained the site of infection. All dogs had patent infections by the time of the second biopsy (S2) even though several from the E litter were amicrofilaremic. Dogs that developed limb edema did so by eight weeks post-infection.

**Spontaneous inflammatory mediator production.** The supernatants from the infected lymph node cultures from the E dogs contained significantly more spontaneously produced HA than the NEa or NEb dogs (P = 0.03) or the uninfected dogs (P = 0.02) at both S1 and S2 (Figure 2). Likewise, the uninfected lymph node cells from the E dogs produced more HA at S1 and S2 than the uninfected dogs (P = 0.04) (Figure 2). At S2 the cells from the uninfected nodes of E dogs spontaneously generated more HA than cells from uninfected nodes of the NE dogs (P < 0.05).

The infected lymph node cells from the E dogs produced more PGE-2 in spontaneous culture at S1 and S2 than the uninfected dogs (P = 0.01) (Figure 3). Similarly, at both S1 and S2 the infected lymph node cells form the NEb litter spontaneously produced significantly more PGE-2 than lymph node cells from uninfected dogs (P = 0.04). The infected lymph node cells from the E dogs spontaneously generated more PGE-2, on average, than the NE dogs at S1, but this difference was not statistically significant. At S2, infected lymph node cells of E dogs produced less spontaneous PGE-2 than at S1, whereas, the NEb dogs produced more PGE-2 than the NEa dogs at both biopsy events (Figure 3).

The cultures from the uninfected lymph nodes showed that at S1 and S2 the E dogs produced more spontaneous PGE-2 than the uninfected control dog lymph node cells (P = 0.04) (Figure 3). The cell supernatants from uninfected nodes of NE dogs produced spontaneous PGE-2 values that were not different at S1 from those of the uninfected dogs, but were significantly higher by S2 (P = 0.02) (Figure 3).

At both time points, the supernatants from infected node cultures of all dogs had more spontaneously produced TNF than the lymph node cells from uninfected dogs (P = 0.01) (Figure 4). There was significantly more TNF produced from infected lymph node cells at S1 compared with S2 for the E and NEb litters only (P < 0.05). The cells from uninfected lymph nodes of all the infected dogs at S1 spontaneously produced more TNF than the uninfected dog lymph node cells (P < 0.01).

**Inflammatory mediator production in response to BpA.** In addition to measuring the production of HA, PGE-2 and TNF produced spontaneously by the lymph node cells, we also measured mediator production of the lymph node cells during incubation with BpA. There were no significant differences in the amount of HA and PGE-2 produced by the infected lymph node cells in response to BpA between any of the litters. We did find that at S1, infected lymph node cells from all dogs, except NEb 1, had slightly increased levels of TNF produced in response to BpA compared with spontaneous levels, but this was not statistically significant on an individual basis (Figure 4). The E dogs at S2, corresponding to the onset of edema, showed a significant increase in the amount of TNF produced in response to BpA (above spontaneous release levels) compared with the NE dogs (P < 0.01) and to themselves at S1 (P < 0.05) (Figure 4).

**Histologic findings.** Hemotoxylin and eosin-stained sections were examined from both infected and uninfected lymph nodes from all dogs at both S1 and S2, as well as lymph nodes from uninfected control dogs. Slides were examined for the presence of neutrophils and eosinophils, as well as for histiocytosis. Paracortical histiocytosis was the most frequently occurring change observed in infected nodes of almost all dogs at the different times post-infection. Histiocytosis was present in the dogs without association either with parasite maturity or biopsy time. Infected node sections from all dogs showed hyperplasia at S1 that, in some cases, was still present at S2. Hyperplasia was present in cortical, paracortical, and/or medullary regions. Eosinophilia was not seen in any of the infected or uninfected lymph nodes at either timepoint in any of the infected dogs. No differences were observed between sections from infected and uninfected dogs in the numbers of neutrophils. There was no correlation between lymph node histologic change and limb edema.

Toluidine blue-staining sections of infected and uninfected lymph nodes were examined for the presence and number of mast cells. Toluidine blue-staining mast cells were count-
ed in 10 high-power fields (hpf) in each section and the mean ± SEM number of mast cells/hpf was calculated. Similar areas within each lymph node (capsule, sinusoids, germinal centers, medulla, and cortex) were counted. Mast cells were found only in the capsule, medullary stroma, and/or the sinusoids. The E dogs did have a higher average number of mast cells (1.4 ± 0.5 mast cell/hpf) than the other dogs, both infected (0.5 ± 0.5 mast cell/hpf) and uninfected (0.5 ± 0.5 mast cell/hpf), but this difference was not statistically significant. The mast cells from the E litter stained very intensely purple and appeared much darker than the majority of mast cells seen in lymph node sections of dogs from other litters. Representative sections from lymph nodes of the E litter as well as NE litters and uninfected dogs lymph nodes were recut and stained as a cohort with toluidine blue a second time to determine if the intensity of toluidine blue staining in the mast cells of the E litter was an artifact of the staining procedure. The mast cells from the recut and stained sections from the E dogs again stained intensely purple compared to the other recut lymph node sections, leading us to believe that the intensity of staining was not due to variation in staining technique.

**Immunohistochemical staining for TNF.** The regional, intranodal distribution, and comparative internodal presence of TNF for individual dogs was evaluated by immunohistochemistry on sections of infected and uninfected lymph nodes. The binding of anti-TNF antibody and subsequent labeling was judged to be specific for TNF present in the lymph node.
sections, based upon the complete absence of staining following addition of recombinant human TNF to the anti-human TNF antibody prior to incubation with lymph node sections.

Infected nodes from all dogs in both the E and NE litters showed strong staining for TNF (seen as red staining) at both timepoints. Infected dogs showed a wider distribution of staining compared with the uninfected lymph node of the same dog (Figure 5A and B). Similarly, the uninfected dogs showed very little TNF staining within the lymph nodes. When anti-TNF staining was found throughout the lymph node, it ranged from very intense in the capsular areas around the afferent lymph vessels, to more diffuse staining in the sinusoidal areas as well as within the germinal centers and secondary follicles. The staining was intercellular and intracellular. Macrophages and mast cells demonstrated intracellular staining with anti-TNF, and extracellular staining was prominent in areas of histiocytosis.

When TNF was present in the uninfected node sections of E and NE dogs, it was restricted to the connective tissue areas (capsule and stroma) and not generally found in the follicles and germinal centers (lymphoid regions). The uninfected control dog sections showed very little labeling with anti-TNF and this was restricted to the capsular and stromal areas.

**In vitro BpA-specific proliferative responses.** For all infected dogs except E3, NEb1 and NEb3, there were changes in the proliferative responses to BpA from S1 to S2 (Figure
Spontaneous TNF Production

![Graphs showing TNF production](image)

**FIGURE 4.** *In vitro* bioactive tumor necrosis factor (TNF) produced in lymph node cell cultures. Lymph node cells (5 × 10⁶/well) were cultured with or without *Brugia pahangi* antigen (BpA) (10 μg/ml) and supernatants harvested after 24 hr in culture. A and B show spontaneously produced levels of TNF by infected (A) and uninfected (B) lymph node cells. C and D shows the response of infected (C) and uninfected (D) lymph node cells to incubation with BpA. Values in C and D represent the average change in response for each litter between spontaneously produced TNF and TNF produced in response to incubation with BpA. Values represent the average for each litter ± standard error. See Figure 1 for additional information.

6). There was no obvious trend in the response of infected lymph node cells to BpA in any of the three litters. All infected lymph node cells did have a significantly higher response to BpA compared with the uninfected dog lymph node cells (*P* < 0.01).

**DISCUSSION**

Experimental infection of dogs with the filarial parasite *B. pahangi* has proven to be an excellent model system to study lymphatic disease pathogenesis associated with lymphatic
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Figure 5. Immunoperoxidase staining of lymph node biopsies for tumor necrosis factor-α (TNF-α). A, infected lymph node from dog NEb1 at S2. Positive TNF staining (red) around the capsular vessel (arrowhead) and within the capsule (arrow) adjacent to the paracortex. B, uninfected lymph node from dog NEb1 at S2 shows very little diffuse capsular staining. (Original magnification × 50.)

Filarialis. Using this model system, we have been able to sequentially biopsy infected and uninfected lymph nodes in dogs selectively bred for increased risk of limb edema following infection. In this study we have described the presence of HA and TNF in infected lymph node cell culture supernatants prior to and at the onset of edema formation. The levels of these two inflammatory mediators were significantly higher in lymph node cultures from dogs genetically at high risk for edema development compared with low-risk infected dogs. The previously reported correlation of episodes of transient edema formation and lymphadenitis with subsequent chronic edema and elephantiasis in humans suggests that defining the etiology of chronic pathology will require knowledge of inflammatory parameters associated with acute edema formation in addition to parameters measured after chronic pathology is established. Once established, chronic pathology is variably associated with adult worm antigenemia. In this report, and in previous reports, acute limb edema formation correlates very strongly with onset of patency in dogs infected with their natural occurring lymphatic filarial.

This is the first report of mastocytosis in peripheral lymph nodes during lymphatic filariasis. The lymph nodes of dogs where three of four siblings developed edema following infection had higher numbers of mast cells in both infected and uninfected lymph nodes compared with lymph nodes from nonedema-forming dogs. It is not clear at this time if the increased intensity of toluidine blue of the mast cells in the E dog lymph node sections is due to increased numbers of granules per cell or due to increased intensity of staining granules possibly due to higher degrees of sulfation of mucopolysaccharide groups within the mast cell granules. Kusche and others found that in rats infected with the nematode Nippostrongylus brasiliensis, compared with uninfected rats, intestinal mast cells stained more intensely with toluidine blue as a result of oversulfated galactoctosaminoglycans.

This is the first report of a familially associated variation in mast cell function as indicated by HA release although antigen non-specific HA release has been shown to correlate with manifestation of atopic dermatitis in dogs and with bronchial asthma in humans. Variations in mast cell function may influence the manifestation of clinical disease in response to this lymphatic filarial nematode. Histamine and other mast cell products that increase vascular permeability may be contributing to the increased interstitial lymph associated with edema formation in these dogs, humans, and even in immunodeficient (nude) mice. The increased vascularity shown to occur locally at the site of infection with Brugia in edema-forming dogs would facilitate HA-dependent edema formation. In addition, mast cell degranulating peptides have been isolated from various nematodes, including filarids, and their presence at the onset of patency may contribute to mast cell degranulation in susceptible individuals. Also, microfilariae of Dirofilaria immitis have been demonstrated to fix complement by the alternative pathway, leading to the generation of the mast cell degranulating anaphylotoxin C3a. Thus, absence of parasite allergen-specific IgE may not prevent limb edema formation in individuals with large populations of mast cells or with highly reactive mast cells at the site of infection.
FIGURE 6. Proliferative response of infected lymph node cells from litter E (A), litter NEa (B), and litter NEb (C). Each graph represents one litter. The proliferative response for *Brugia pahangi* antigen–stimulated (10 μg/ml) (□) cells are superimposed on unstimulated cultures (□) at day 4 or day 6 at both timepoints (S1 and S2). Data shown as mean counts per minute of triplicate cultures. See Figure 1 for additional information.
Tumor necrosis factor levels in the E dogs were higher in the infected node cell than in uninfected node cell cultures and more frequently responded to parasite antigen addition to culture than dogs from litters not showing frequent edema formation. A recent study of humans with Bancroftian filariasis showed significantly elevated serum levels of TNF-α in patients with acute disease and not in microfilaria carriers or in patients with chronic disease. Histiocytosis that is present in human lymphatic filariasis was also noted in all infected lymph nodes in this study and may contribute to elevated levels of TNF. Tumor necrosis factor is produced by several cell types, including macrophages and mast cells, and was most clearly associated here with an early response to infection and not as closely associated with edema formation as HA release. Histamine release being more restricted to mast cells suggests a prominent role for mast cell releasability in risk of edema formation.

The PGE-2 levels were elevated in infected dogs but did not show any clear trend regarding association with edema formation. There were two exceptions to the familial association of limb edema manifestation. When high levels of HA and PGE-2 were produced by infected lymph node cells from dog NEb5, this dog did develop edema in contrast to his littermates. On the other hand, dog E1 in the edema forming litter did not have edema yet had levels of PGE-2 and HA that were comparable with those of his littermates, indicating that there may be other ameliorating factors involved in the development of limb edema. These results indicate that multiple factors may be working in synergy or alone to produce edema, but elevated HA and TNF release in the absence of antigen as well as elevated TNF levels in response to antigen were characteristic of dogs from the litter with high potential for edema formation. If inherited traits in mast cell function are shown to include increased releasability of HA, then it would be important to determine whether or not this trait correlates with lymphatic filarial disease in humans.

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