

EARLY TISSUE MIGRATION OF AND HOST RESPONSE
TO *BRUGIA PAHANGI* IN GERBILS

A Thesis

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Table of Contents

List of Tables.....	iii
List of Figures	iv
Abstract.....	v
Introduction and Literature Review	1
Materials and Methods.....	8
Gerbils and Parasites	8
Larval Migration	8
Histology	8
Cytokine Quantitation	9
Primers and Probes.....	10
Statistics	11
Results.....	13
Larval Migration	13
Histology	13
Cytokine mRNA Quantitation.....	16
Discussion and Conclusions.....	23
References	32
Vita.....	37

List of Tables

1. Primers and probes used for RT-PCR (TaqMan) for interleukin 6 and tumor necrosis factor 12
2. Mean and percent larval recoveries from tissues of necropsied gerbils ID inoculated with *B. pahangi* L3 in the left hindlimb 14
3. Location of larvae and presence or absence of inflammation in tissues recovered from gerbils ID inoculated with *B. pahangi* L3 in the left hindlimb 15

List of Figures

1. *Brugia pahangi* larvae in the dermis of the left hindlimb of a gerbil 3 hours after ID infection with 100 *B. pahangi* L3 17
2. *Brugia pahangi* larvae within a deep dermal lymphatic vessel of a gerbil 3 days after ID infection with 100 *B. pahangi* L3 18
3. *Brugia pahangi* larvae in the subcapsular sinus of the left popliteal lymph node of a gerbil 7 days after ID infection with 100 *B. pahangi* L3..... 18
4. Quantitation of IL-6 mRNA in spleens (A), renal lymph nodes (B), and popliteal lymph nodes (C) 19
5. Quantitation of TNF mRNA in spleens (A), renal lymph nodes (B), and popliteal lymph nodes (C) 20
6. Quantitation of IFN gamma mRNA in spleens (A), renal lymph nodes (B), and popliteal lymph nodes (C) 21
7. Quantitation of IL-4 mRNA in spleens (A), renal lymph nodes (B), and popliteal lymph nodes (C) 22

Abstract

The host-parasite interaction during early filarial nematode migration is poorly understood. The objective of this study was to develop a model of early cutaneous filarid migration using *Brugia pahangi* in the jird (gerbil) host and measure the histologic and cytokine responses during this period. Male gerbils were intradermally inoculated in the left hindlimb with 100 *B. pahangi* L3 then necropsied at 3 hours, 24 hours, 3 days, 7 days, and 28 days post-infection. Larvae were recovered and tissues collected for histology and cytokine measurement. At 3 hours, most larvae (96.3%) were recovered from tissues associated with the infection site. Migration away from the infection site occurred within 24 hours. By 7 days, larvae were dispersed throughout the lymphatic system, including the spermatic cord lymphatics. Larvae were identified on histologic exam at all time points and were located in the dermis, muscle, lymphatic vessels, and lymph nodes. Predominantly neutrophilic inflammation was frequently present around larvae in the dermis and muscle at 3 and 24 hours. Levels of the cytokines IL-6, TNF, IFN-gamma, and IL-4 were measured in the spleen and popliteal and renal lymph nodes. IL-6 and TNF both showed a peak at 3 hours followed by consistent decline in all tissues. No clear increase in expression was appreciated for IFN-gamma. IL-4 remained low through 7 days and rose by 28 days in all tissues. These results indicate the ability of filarid L3 to rapidly migrate through host tissue and they support intradermal gerbil infection as a model for early filariasis. Cytokine analysis and histology indicated an acute host inflammatory response following initial infection, with Th2 polarization occurring later in the course of infection.

Introduction and Literature Review

Lymphatic filariasis is a mosquito-borne disease and one of the oldest diseases known to man, with recorded descriptions dating as far back as 70 AD (Scott 2000). Today, it is estimated that there are approximately 128 million cases of lymphatic filariasis and approximately 751 million people living in endemic areas in which transmission has been shown to occur (Scott 2000). Most cases of lymphatic filariasis occur in Southeast Asia, the Indian subcontinent, and Africa. Three species of filarid nematodes localize in human afferent lymphatics and are associated with development of the pathologic lesions of lymphatic filariasis: *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*. Filarid microfilariae enter the midgut of a mosquito vector after a blood meal then migrate to the thoracic musculature and develop into 3rd stage larvae (L3). L3s then migrate to the head tissues and proboscis of the mosquito where they can be transmitted to a vertebrate host upon mosquito feeding (Bartholomay *et al.* 2002). During feeding, L3s are deposited on the skin of the host in a drop of haemolymph and migrate into the skin of the host through the puncture wounds made by the mosquito vector (Ewert 1967). The larvae then make their way to the local afferent lymphatics. Here they molt to 4th stage larvae at approximately 7 days post-infection and to adult worms at approximately 30 days post-infection. Microfilariae are evident in circulation by 60 days post-infection. The host response to the presence of parasites in the lymphatics and parasite products in host tissues results in lymphatic dilation and formation of granulomatous lesions in and around lymphatics and lymph nodes (Vincent *et al.* 1980, Klei *et al.* 1981, von Lichtenberg 1987, Jeffers *et al.* 1987, Klei *et al.* 1990).

In endemic regions of lymphatic filariasis, humans exhibit a broad range of responses to infection. These responses are classically divided into three main groups: 1) individuals with no clinical disease symptoms and no microfilaremia (immune or occult infection), 2) individuals with microfilaremia but few or no disease symptoms (immunologically hyporesponsive), and 3) those individuals with symptoms of either acute or chronic lymphatic pathology (Nutman *et al.* 1995). Likewise, there is also a broad spectrum of clinical presentation of lymphatic filariasis (Kumaraswami 2000). Variation in disease depends on the infecting nematode species and the geographic region of transmission, as well as the actual patient. At one end of the spectrum are the majority of people residing in endemic areas, termed immunologically hyporesponsive. These individuals have large numbers of microfilariae with only subclinical signs of diseases. Lymphatic pathology (tortuous lymphangiectasia) and even mild renal damage (microscopic hematuria and proteinuria) are present, but only detectable through ultrasound and/or microscopic tissue examination. Since people in this category of disease have persistently circulating microfilariae, they act as a disease reservoir in the population. At the other end of the disease spectrum are individuals that show overt signs of disease and are typically microfilaria negative. These individuals represent the “classic” picture of clinical lymphatic filariasis. Both acute and chronic disease manifestations are described. Acute disease is characterized by recurrent fevers and adenolymphangitis. Attacks of fever are a more prominent feature in *Brugia* infections. Lymphatic obstruction, lymphedema, and elephantiasis are characteristic of chronic disease (Kumaraswami 2000).

Many laboratory animal models have been developed to answer questions regarding filarial-induced pathology including cats, dogs, ferrets and non-human primates. Most significant among these have been the mouse, rat, and Mongolian gerbil (*Meriones unguiculatus*, also known as a jird). Since *Wuchereria* does not develop within non-primate species, *B. malayi* and *B. pahangi* are the filarid species most typically examined under laboratory conditions. Immunologically intact mice are non-permissive to infection with *Brugia*. Larvae can be maintained for short time periods, but the parasite is usually eliminated after the molt to L4. Nude and SCID mice will support infection, though it has recently been shown that SCID mice require the presence of natural killer cells for development of *B. malayi* infection (Babu *et al.* 1998). Furman and Ash (1983) demonstrated that *B. pahangi* could develop to adult parasites in neonatal mice, though the adult worms were smaller in length than worms of comparable age in jirds and cats. Since wild-type, adult mouse strains show resistance to filarid infection, the applicability of the mouse model to the human condition may be questioned. The main advantage of the mouse model over many other laboratory species, however, is the wide availability of inbred strains and immunologic reagents.

The rat model shares the advantage of reagent and strain availability. Several studies have investigated the susceptibility of various rat strains to filarid infection. Similar to mice, immunologically altered rats, specifically athymic nude rats, had higher percentages of adult worm recovery and microfilaremic (mf+) *B. pahangi* infections as compared with wild-type rats (Lawrence *et al.* 1992). In immunologically intact rats the percentage of mf+ infections varies with rat strain and parasite dose. For example, Lawrence and Denham (1991) found that 56% of adult PVG mice became mf+ when

infected with 100 *B. pahangi* L3, but 94% became mf+ when infected with 500 larvae. A summary of rat responses was examined by Bell, *et al.* (1999) by infecting various doses of *B. pahangi* L3 into 8 different inbred strains of suckling, weanling, and adult rats. In this study, it was shown that a single infection of any of the 8 examined strains produced rats in three categories: mf+ infection, occult infection, and cleared primary infection. The proportions of animals falling into the three categories varied with strain, sex, age, and parasite dose with moderate infections (100-200 L3) in adult, male, Lewis or WKA rats producing the highest patency rates (72% and 73%, respectively).

Mongolian gerbils are highly permissive to infection with both *B. malayi* and *B. pahangi*. They support full development of Brugian parasites and lack the variability seen with the rat model. Jirds have been used extensively in the study of lymphatic filariasis (Jeffers *et al.* 1987, Klei *et al.* 1988, McVay *et al.* 1990). Infection of jirds with *Brugia* L3 results in chronic infections characterized by persistent microfilaremia and chronic, subclinical lymphatic and lymph node lesions - lymphatic dilation, granulomatous lymphatic thrombi, and granulomatous lymphadenitis (Klei, *et al.* 1988). In these respects, the jird is similar in disease manifestation to the “immunologically hyporesponsive” state which represents the largest part of endemic human infection. The primary disadvantage of the jird has been the lack of available standardized immunological reagents. Recent advances in cloning genes encoding for several key gerbil cytokines has improved this situation (Gaucher *et al.* 2001, Chirgwin *et al.* 2002).

The chronic state of filarid infection is characterized by the presence of adult worms within the host lymphatic system and down-regulation of the host immune response, specifically decreased lymphocyte proliferation and decreased production of

IFN γ and IL-2 in response to filarial antigens (King 2002). Most filarial research has focused primarily on the pathology and immune regulation of the chronic state of infection, though a few previous studies have examined the events of early filarid infection, including early larval migration and host immunologic response. The earliest studies examined transfer of the filarid parasite to the mammalian host. Ewert (1967) visualized the feeding of *Aedes togoi* and *A. aegypti* mosquitoes infected with *B. pahangi* on laboratory mice. Ewert's study revealed that the filarid larvae were deposited on the skin of the host and then migrated into the dermis through the puncture wound made by the feeding mosquito. Larvae were identified histologically in the dermis within 2-5 minutes of deposition of larvae on the skin. A similar study by Gordon and Crewe (1953) examined the deposition of *Loa loa* by *Chrysops silacea*, showing that damage to host skin by the vector was necessary for larval skin penetration. These studies focused primarily on the vector portion of filarial infection. Other studies have tried to elucidate the actions of the parasite within the host.

Much of this parasite migration work has been conducted in gerbils. Ash and Riley (1970) documented the development of both *B. malayi* and *B. pahangi* in the gerbil. Third stage *Brugia* larvae (L3) were subcutaneously inoculated into gerbils. Patency of infection was tracked and larvae were recovered from various tissues between 6 and 26 days post-inoculation. Larvae were recovered from the heart, lungs, viscera, lymph nodes, subcutaneous fat, and pelt by day 6 (*B. pahangi*) or day 7 (*B. malayi*) post-inoculation. Ah and Thompson (1973) also examined development of *B. pahangi* infection in gerbils. After subcutaneous injection of *B. pahangi* L3, small numbers of larvae were recovered from regional lymphatics as early as 2.5 days post-inoculation. At

this time, over 50% of the larvae were recovered from the skin around the inoculation site. The percentage of larvae found around the infection site progressively declined to 20% at day 30 post-inoculation. The data from this study is, however, based on the recoveries from one animal per time point. The migratory ability of inoculated *B. pahangi* L3 was further demonstrated by Ah *et. al.* (1974) using ocular inoculation. Infective *B. pahangi* L3 in suspension were dropped onto the eyeballs of both gerbils and dogs. Gross examination revealed that some larvae had migrated to the heart and lungs within 5 minutes of inoculation, and larvae were found histologically in the afferent vessels of the cervical lymph nodes by 2 hours post-inoculation.

Work has also been conducted using other host-parasite species models. The number and distribution of *B. pahangi* larvae in infected cats was examined by Suswillo *et. al.*(1982). Within three hours of infection of the hindlimbs of cats, approximately 50% of the recovered larvae were located in the regional lymph node (popliteal lymph node and perinodal sinus) indicating rapid larval migration. A study tracking migration of *Monanema martini* in the striped grass mouse (*Lemniscomys striatus*), a model system for human Onchocerciasis, also demonstrated rapid larval migration. After subcutaneous inoculation of *M. martini* L3, larvae were recovered from regional lymph nodes (lumbar and mesenteric lymph nodes) within 6 hours post-infection (Wanji *et al.* 1990). Longer migration times are seen with *Dirofilaria immitis*. In beagles inoculated with *D. immitis* L3, larvae were recovered only from the skin and muscle through the first 58 days of infection (Kotani *et al.* 1982). Similar findings are reported in *D. immitis* inoculated ferrets, with larvae recovered primarily from the muscle and subcutis through day 91 post-infection (Supakorndej *et al.* 1994).

Only recently has filarial research begun to turn toward the early host response portion of the host-parasite interaction. Babu and Nutman (2003) have developed an *in vitro* model system to study the early host immune response to filarid infection using human peripheral blood monocytes (PBMC) and *B. malayi* L3. Their work shows that expression of proinflammatory Th1 cytokines is significantly increased in PBMC within 24 hours of exposure to live *B. malayi* L3. Similarly, *in vivo* studies of mice indicate an influx of neutrophils within 24 hours of intraperitoneal infection of mice with either *B. malayi* or *B. pahangi* L3 (Ramalingam *et al.* 2003, Rajan *et al.* 2002). Semnani *et al.* (2004) have shown that within 72 hours of exposure to live *B. malayi* L3, human epithelial-derived Langerhans cells experience increased migration from the epidermis and a down-regulation of genes involved in antigen presentation.

Though the above-described research has examined various aspects of early filarid infection, detailed information concerning the events of the host response to the first few hours and days of filarid infection is lacking. The aim of this study was to establish a quantitative *in vivo* model of filarid infection to begin to more fully characterize the host immune response to filarid migration and to determine the possible significance of that response on establishment and course of infection. To that end, a *B. pahangi* -jird system using intradermal larval inoculation was developed. Since L3 migrate into the dermis of the host via the puncture wound created by a feeding mosquito, intradermal inoculation was selected, rather than the commonly used subcutaneous and intraperitoneal routes, to more accurately represent the natural route of larval migration. The developed model was then used to quantitate parasite recoveries, measure cytokine levels, and examine histologic lesions over time.

Materials and Methods

Gerbils and Parasites. Male Mongolian gerbils (*Meriones unguiculatus*) approximately 8 weeks of age were obtained from Charles River (Wilmington, Mass.) and were maintained on standard rodent chow and water *ad libitum*. *Brugia pahangi* third-stage larvae (L3) were recovered from infected *Aedes aegypti* (Black eye strain) mosquitoes by use of a Baermann procedure. Motile L3 were placed into HEPES-buffered RPMI 1640, pH 7.2 (Gibco Laboratories, Grand Island, NY) containing penicillin (100 U/ml), streptomycin (100 µg/ml), and Amphotericin B (0.025 µg/ml). All infections were given by intradermal (ID) injection using a 28 gauge needle into the medial aspect of the distal hindlimb and consisted of *B. pahangi* L3 suspended in 50 µl of RPMI. Control (uninfected) animals received ID injections of 50 µl of RPMI from the final larval wash after Baermannization.

Larval Migration. Gerbils were infected in the left distal hindlimb with 100 *B. pahangi* L3, then selected at random and necropsied at 3 hrs, 24 hrs, 72 hrs, 7 days, and 28 days post-infection (DPI). Six gerbils were necropsied at each time point. Larvae were recovered from the animals by dissection and teasing of the left (injected) hindlimb skin and muscle, major lymph nodes and lymphatics, heart, lungs, testicles, and spermatic cords. Tissues were then soaked in phosphate-buffered saline (PBS) for at least 1 hour before being carefully dissected with forceps and larvae recovered with the aid of a stereomicroscope. Larval recoveries from the skin and muscle of the left hindlimb were pooled.

Histology. Fifteen gerbils were ID infected with 100 *B. pahangi* L3 in the left distal hindlimb. Five control gerbils were injected with 50 µl of RPMI from the final larval

wash after Baermannization in the left and right distal hindlimbs. Immediately after infection (0 hr) and at 3 hrs, 24 hrs, 72hrs, and 7 DPI, 3 infected animals and one control animal were selected at random, euthanised, and the hindlimbs (intact), renal lymph nodes (RLN), popliteal lymph nodes(PLN), and subinguinal lymph nodes removed. Tissues were immersed in 10% neutral buffered formalin and allowed to fix for a minimum of 7 days. After fixation, hindlimbs were deboned and cut into 5 to 7 cross-sectional pieces. Tissue pieces were embedded in paraffin. Two sections, 10 µm apart, were cut from the anterior face of the paraffin block and then two sections were cut from the posterior face of the paraffin block. Lymph nodes were embedded intact in paraffin and 2 to 4 serial sections made. All sections were stained with hematoxylin and eosin.

Cytokine Quantitation. Gerbils were divided into two groups. One group was ID infected with 200 *B. pahangi* L3: 100 L3 in the right hindlimb and 100 L3 in the left hindlimb. The second (control) group was intradermally injected in the right and left distal hindlimbs with RPMI from the final larval wash after Baermannization. At 3 hrs, 24hrs, 72hrs, 7 days, and 28 DPI, 6 infected and 2 control animals were selected at random, euthanised, and the spleen, RLN, and PLN were collected for cytokine quantitation. Single cell suspensions were prepared by passing tissues through a Falcon 70 µm cell strainer (BD-Biosciences, Paramus, New Jersey). Suspensions were centrifuged at 1200 RPM for 15 minutes at 4°C. Pellets were resuspended and homogenized in 0.5 ml RNAMStat 60 (Tel-test, Friendship Tex.) and stored at -70°C.

RNA was isolated from the RNAMStat samples using a chloroform extraction as per manufacturers instructions (Tel-test). RNA (1200 ng per reaction) was then reverse transcribed in the presence of Oligo dT primers to produce single strand cDNA. The

cDNA was used as a template in quantitative PCR using the ABI PRISM 7700 sequence detection system (TaqMan; Applied Biosystems, Foster City, Calif.) for quantitation of hypoxanthine phosphoribosyltransferase (HPRT) (GenBank accession no. L37778), interleukin 4 (IL-4) (accession no. L37779), interferon gamma (IFN γ) (accession no. L37782), tumor necrosis factor (TNF) (accession no. 171082), and interleukin 6 (IL-6) (accession no. AY570509).

TaqMan PCRs were performed in duplicate and conducted using Applied Biosystems Universal PCR master mix according to the manufacturer's directions. Relative standard curves were constructed from cDNA prepared from RNA isolated from stimulated gerbil spleen cells. Spleens from non-infected male gerbils were harvested into warm RPMI 1640 supplemented with 2% heat-inactivated fetal calf serum (FCS) and single cell suspensions obtained. Cells were tested for viability by Trypan blue exclusion and adjusted to a final concentration of 1×10^6 cells/ml. Cell suspensions were placed into culture wells, incubated at 37°C, and stimulated with either concavalin A (10 μ g/ml) for 24 hrs. or lipopolysaccharide (LPS) (10 μ g/ml) for 24 or 48 hrs. The concavalin A-stimulated cDNA was used as a standard curve for IL-4 and IFN γ measurements, and the LPS - stimulated cDNA was used as a standard curve for TNF and IL-6 measurements. All data are presented as mean fold change of a treatment group compared to the mean value for the appropriate control group \pm standard error.

Primers and Probes. Primers based on the rat IL-6 sequence were used to amplify IL-6 from gerbil cDNA. RNA was isolated and cDNA prepared from gerbil spleen cells stimulated with LPS for 24 hrs as described above. This cDNA was used as a template in PCR conducted using the Advantage 2 Taq system (Clontech, Palo Alto, Calif.) and the

following conditions: 94°C for 30 s, then 25 cycles of 94°C for 20 s, 61°C for 30 s, and 68°C for 45 s. A PCR product of 581 base pairs was cloned using the TOPO blunt cloning system (Invitrogen) and sequenced. The product showed 84-86% homology with rat IL-6 at the nucleotide level.

Oligonucleotide sequences for HPRT, IL-4, and IFN γ have been previously described (Chirgwin 2002). Primer and probe sequences for IL-6 and TNF are listed in Table 1. All gerbil, gene-specific primers and probes were generated commercially (GeneLab, Baton Rouge, La; Applied Biosystems, Foster City, Calif.). Primers were unlabeled, and probes were labeled with the reporter dye 6-FAM (6-carboxyfluorescein) and the quencher dye TAMRA (6-carboxytetramethylrhodamine).

Statistics. All statistical analyses were performed using SigmaStat (SSPS Inc., Chicago, Ill.). Larval recoveries and cytokine mRNA data were analyzed by Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks with subsequent pairwise comparisons analyzed using either Dunn or Tukey test.

TABLE 1. Primers and probes used for RT-PCR (TaqMan) for interleukin 6 (IL-6) and tumor necrosis factor (TNF)

Gene	Sense (5'-3')	Antisense (3'-5')	Probe sequence *
IL-6	AACACCAAAACCCTAATTCGTATCTT	GCCTCGGAAGTTGGTCA	AACAAGAGGTGAAGGATCCAGG TCAAATAGTCTTT
TNF	TCAGAACGCCAGCGACAA	CAGCTGCTCCTCCACTTGGT	CTGTGGCCCATGTCGTACCCAA

* Probe sequences were labeled with the reporter dye FAM on the 5' end and the quencher dye TAMRA on the 3' end.

Results

Larval Migration. Gerbils were inoculated with *B. pahangi* L3 and larvae recovered at various time points after infection. Larvae were recovered from the skin and muscle of the inoculated hindlimb, popliteal, renal, and subiliac lymph nodes, heart, lungs, testicles, and spermatic cords. The carcass was also soaked in RPMI after removal of the organs ('body soak') and larvae recovered. At 3 hours post-infection (p.i.), 96.3% of recovered larvae were present in the tissues (skin and muscle) associated with the infection site. By 24 hours p.i., 53.2% of larvae were recovered from tissues of the infection site. At 3 and 24 hrs., significantly ($P < .05$) more larvae were present in the skin and muscle surrounding the infection site than in other examined tissues; however, larvae had migrated away from the infection site and had become established within the lymphatics, including spermatic cord lymphatics by 24 hrs. (Table 2). Larvae were well established in the lymphatic system by 28 days p.i. with the majority of larvae located in the lymphatics associated with the spermatic cord (35.7%) (Table 2). The locations of the recovered larvae indicate a pattern of progressive migration into the lymphatic system away from the site of initial larval entry.

Histology. Larvae were visualized in the skin, muscle, lymphatic vessels, lymph nodes, and veins (Table 3). Immediately after larval inoculation (0 hours p.i.), larvae were visualized in the skin around the injection site. Multifocal, moderate dermatitis and rare, mild myositis were noted at 3 hours and 24 hours p.i.. The inflammatory infiltrate was comprised of neutrophils with mild to moderate numbers of eosinophils, occasional basophils, and rare mononuclear cells. Larvae visualized within the dermis were often surrounded by the inflammatory infiltrate (Figure 1), but not all areas of inflammation

TABLE 2. Mean and percent larval recoveries from tissues of necropsied gerbils ID inoculated with *B. pahangi* L3 in the left hindlimb. Values represent the mean \pm SD and percent of total larvae recovered by tissue type.

Tissue	Time Post-infection									
	3 hours		24 hours		3 days		7 days		28 days	
	Mean \pm SD	%	Mean \pm SD	%	Mean \pm SD	%	Mean \pm SD	%	Mean \pm SD	%
Hindlimb- skin,muscle	33.8 \pm 18.5 ^a	96.3	11.0 \pm 7.0 ^a	53.2	4.3 \pm 4.9	13.7	7.3 \pm 5.3	28.8	0.7 \pm 1.2	5.9
Popliteal lymph node	0.8 \pm 0.9	1.8	3.2 \pm 3.4	14.7	7.3 \pm 4.6	38.4	3.3 \pm 2.1	15.2	1.3 \pm 1.0	9.3
Subiliac lymph node	0.2 \pm 0.4	0.3	2.2 \pm 2.1	8.4	3.8 \pm 4.8	16.6	3.7 \pm 3.2	13.1	0.8 \pm 1.2	4.9
Renal lymph node	0.7 \pm 0.8	1.3	3.5 \pm 2.8	15.5	6.8 \pm 7.5	17.8	7.0 \pm 4.0	26.3	1.8 \pm 1.2	10.1
Spermatic cord	0	0	0.7 \pm 0.8	2.4	1.5 \pm 2.5	3.1	1.8 \pm 3.1	6.6	8.2 \pm 7.3	35.7
Testicle	0	0	0.2 \pm 0.4	0.7	0.3 \pm 0.5	0.7	1.2 \pm 1.6	6.2	2.8 \pm 4.2	8.7
Heart	0	0	0.3 \pm 0.5	1.0	0.6 \pm 1.0	2.1	0.3 \pm 0.5	1.8	2.7 \pm 4.7	8.3
Lung	0	0	0.3 \pm 0.8	1.1	0.3 \pm 0.5	1.0	0	0	1.2 \pm 1.2	5.3
Body soak	0.2 \pm 0.4	0.3	0.7 \pm 0.5	3.0	2.2 \pm 2.2	6.5	0.7 \pm 0.8	3.0	1.5 \pm 1.6	10.6
Total # larvae	35.7 \pm 20.2		22.0 \pm 10.8		27.3 \pm 17.7		25.2 \pm 6.7		21.7 \pm 15.2	

^a mean larval recoveries in the hindlimb are statistically significant from recoveries in remaining tissues at 3 hrs. and 24 hrs. at P< .05.

TABLE 3. Location of larvae and presence or absence of inflammation in tissues recovered from gerbils ID inoculated with *B. pahangi* L3 in the left hindlimb. Tissues were formalin fixed and sectioned for histologic exam. Sections were stained with hematoxylin and eosin. Control animals were inoculated with larval wash media

Time post-infection	Tissue							
	Hindlimb (skin, muscle)		Popliteal lymph node		Renal lymph node		Subiliac lymph node	
	Inflammation	Larvae	Inflammation	Larvae	Inflammation	Larvae	Inflammation	Larvae
Control	—	—	—	—	—	—	—	—
0 hrs.	—	+	—	—	—	—	—	—
3 hrs.	+	+	—	+	—	—	—	—
24 hrs.	+	+	—	+	—	+	—	+
3 days	+	+	—	+	—	+	—	+
7 days	+	+	—	+	—	+	—	+

contained identifiable parasite material. Occasional larvae without associated inflammation were also noted. At 3 days and 7 days p.i., inflammatory foci were occasionally noted, but most larvae had no associated inflammation. When present, inflammation at 3 and 7 days consisted primarily of macrophages, lymphocytes, and occasional plasma cells with lesser numbers of neutrophils and eosinophils. Larvae identified within the vascular system (lymphatics and veins) were not associated with an intravascular inflammatory response (Figure 2), but occasional perilymphatic inflammation was seen. Within lymph nodes, larvae were most frequently located in perinodal lymphatics and capsular sinuses with occasional larvae noted within nodal parenchyma (Figure 3). When compared with control lymph nodes, no significant lymphadenitis was appreciated. All identified larvae appeared intact and non-degenerate. No significant inflammation was noted in any of the control tissues injected with the final larval wash media (post-baermannization).

Cytokine mRNA Quantitation. Cytokine mRNA levels were quantitated in the spleens, renal lymph nodes, and popliteal lymph nodes collected from gerbils necropsied at various time points after infection. In all tissues, IL-6 (Figure 4) and TNF (Figure 5) levels both exhibit a peak at 3 hours p.i. followed by a rapid decline. The IL-6 levels at 3 hours were significantly higher ($P < .05$) than levels at later time points in the spleen (7D), RLN (3D and 28D), and PLN (28D) (Figure 4). Levels stay consistently low through 28 days p.i. with the exception of a rise in IL-6 in the spleen at 28 days p.i.. This value was not significantly different from the 3 hour, 24 hour, or 3 day levels ($P < .05$, Figure 4A). In all tissues and at all time points, IFN- γ levels were low and exhibited no clear increase in expression (Figure 6). IL-4 levels were low through 7 days p.i. then peaked at 28 days

p.i. all tissues (Figure 7). A peak in IL-4 levels was also noted at 24 hours p.i. in the spleen, however this value was not significantly different from the 3 day or 28 day levels ($P < .05$, Figure 7A).

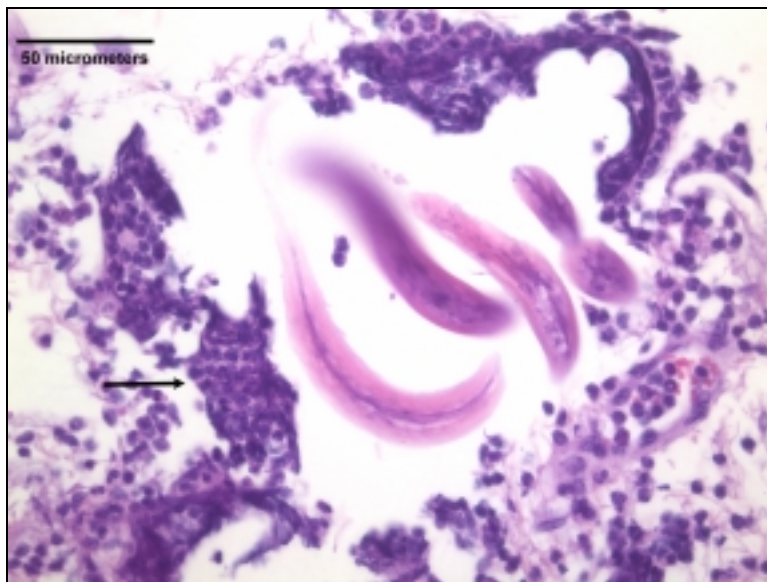


FIGURE 1. *Brugia pahangi* larvae in the dermis of the left hindlimb of a gerbil 3 hours after ID infection with 100 *B. pahangi* L3. The arrow indicates inflammatory infiltrate surrounding the larvae that consists primarily of neutrophils with lesser numbers of eosinophils, basophils, and mononuclear cells. Magnification = 40X

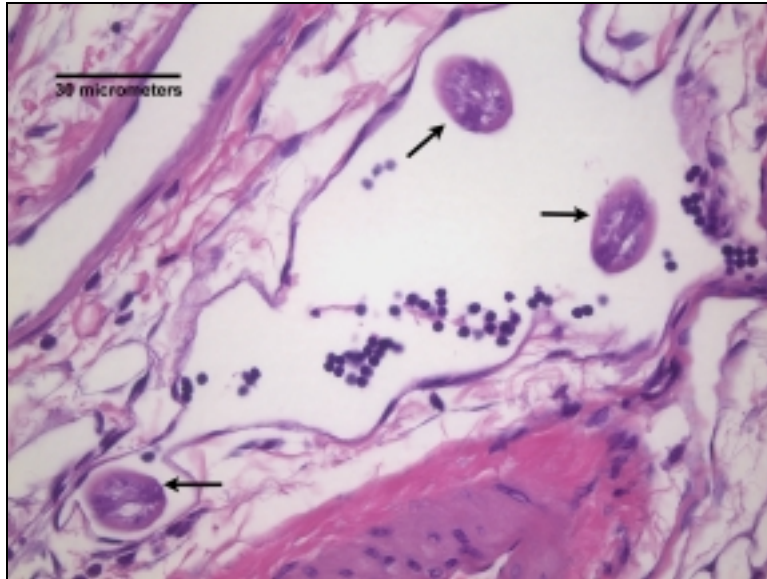


FIGURE 2. *Brugia pahangi* larvae within a deep dermal lymphatic vessel of a gerbil 3 days after ID infection with 100 *B. pahangi* L3. Arrows indicate larvae within the lymphatic vessel. Magnification = 40X

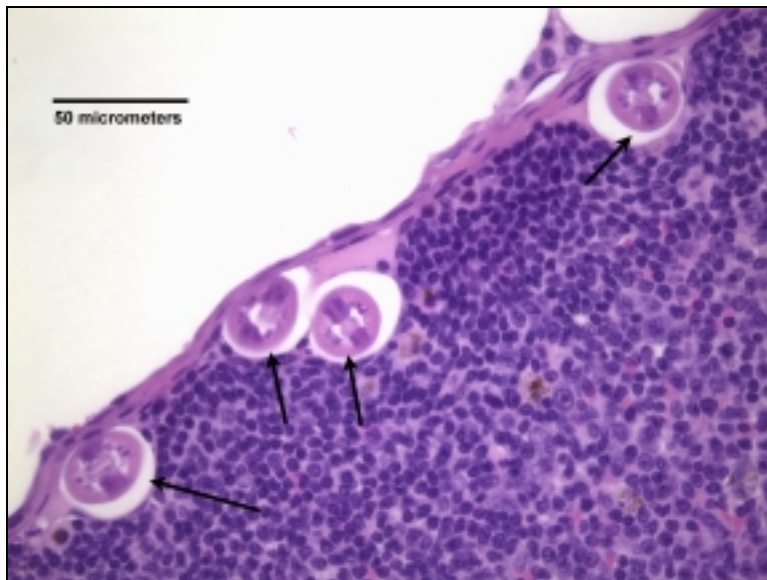


FIGURE 3. *Brugia pahangi* larvae in the subcapsular sinus of the left popliteal lymph node of a gerbil 7 days after ID infection with 100 *B. pahangi* L3. Arrows indicate larvae within the nodal sinus. No significant inflammation was appreciated within the lymph node. Magnification = 40X.

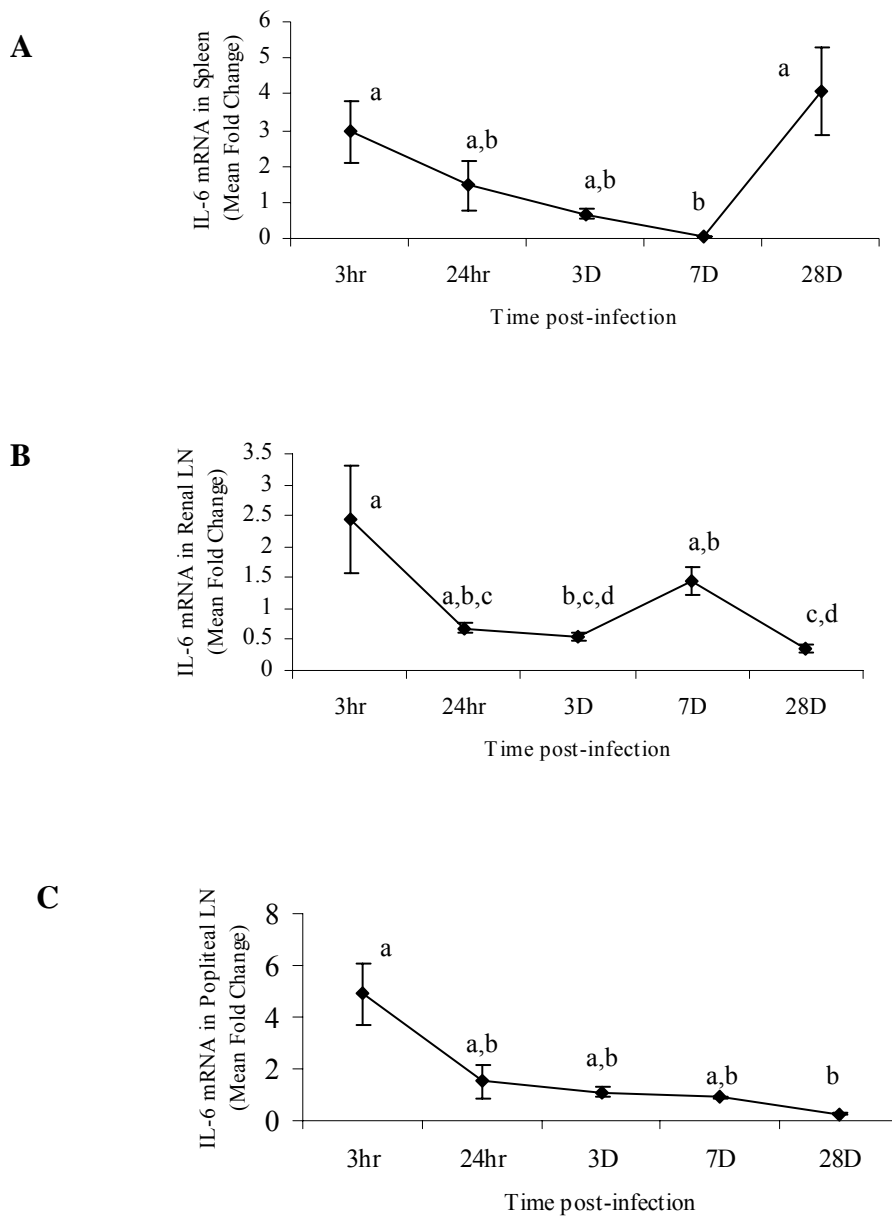


FIGURE 4. Quantitation of IL-6 mRNA in spleens (A), renal lymph nodes (B), and popliteal lymph nodes (C). Gerbils were necropsied at 3 hours, 24 hours, 3 days, 7 days, and 28 days after ID inoculation with *B. pahangi* L3. mRNA levels were measured by RT-PCR and values are expressed as mean fold change as compared with control animals. Superscript letters indicate statistical significance ($P < .05$). Values with the same letter are not statistically different.

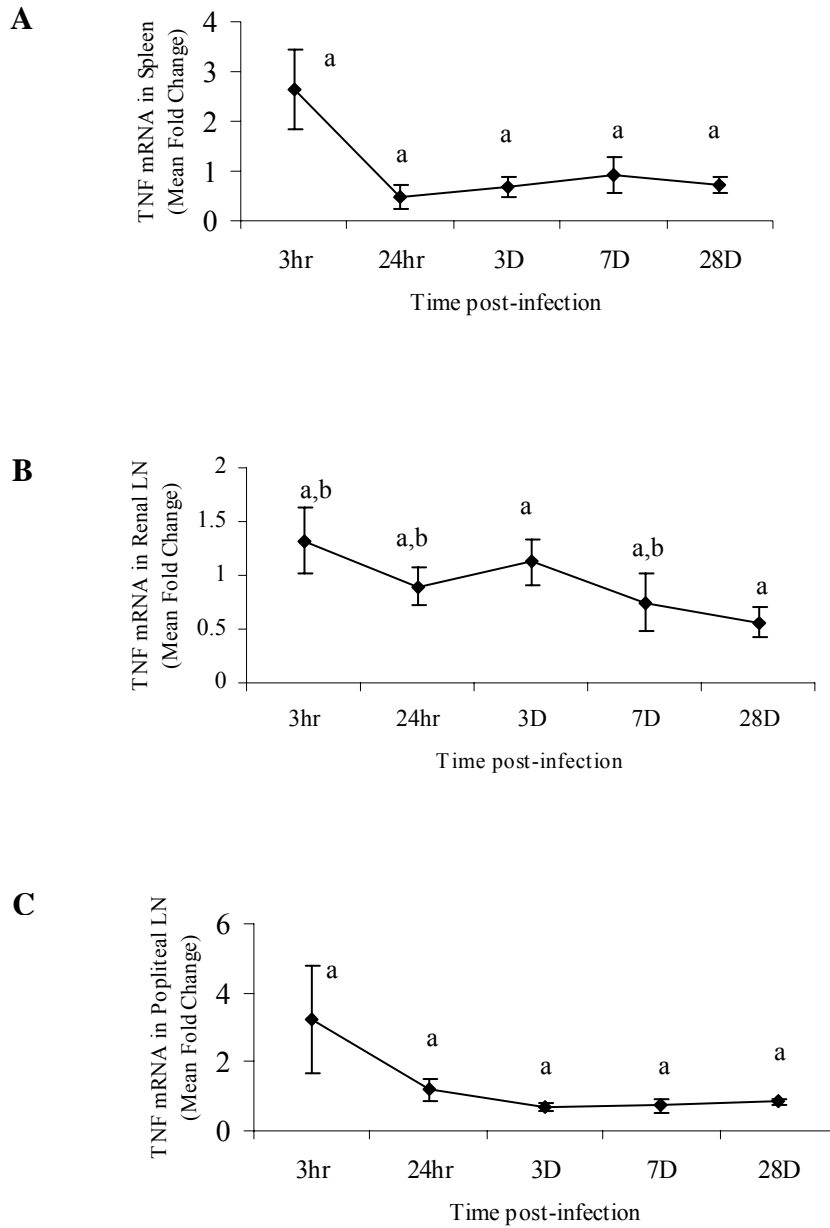


FIGURE 5. Quantitation of TNF mRNA in spleens (A), renal lymph nodes (B), and popliteal lymph nodes (C). Gerbils were necropsied at 3 hours, 24 hours, 3 days, 7 days, and 28 days after ID inoculation with *B. pahangi* L3. mRNA levels were measured by RT-PCR and values are expressed as mean fold change as compared with control animals. Superscript letters indicate statistical significance ($P < .05$). Values with the same letter are not statistically different. No statistically significant differences were found in the spleen ($P = .065$) or popliteal lymph node ($P = .2$).

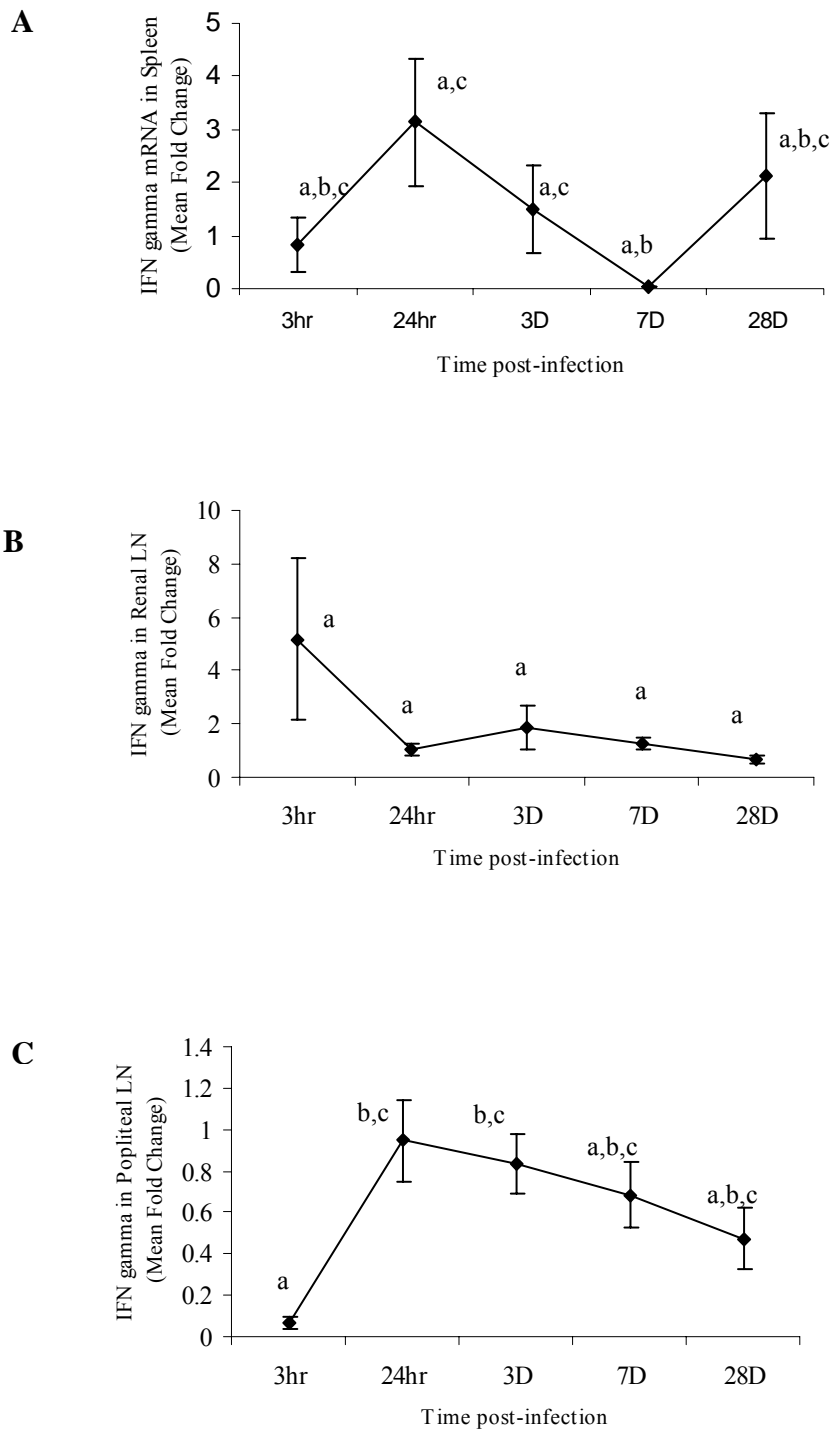


FIGURE 6. Quantitation of IFN gamma mRNA in spleens (A), renal lymph nodes (B), and popliteal lymph nodes (C). Gerbils were necropsied at 3 hours, 24 hours, 3 days, 7 days, and 28 days after ID inoculation with *B. pahangi* L3. mRNA levels were measured by RT-PCR and values are expressed as mean fold change as compared with control animals. Superscript letters indicate statistical significance ($P < .05$). Values with the same letter are not statistically different. No statistically significant differences were found in the renal lymph nodes ($P = .094$)

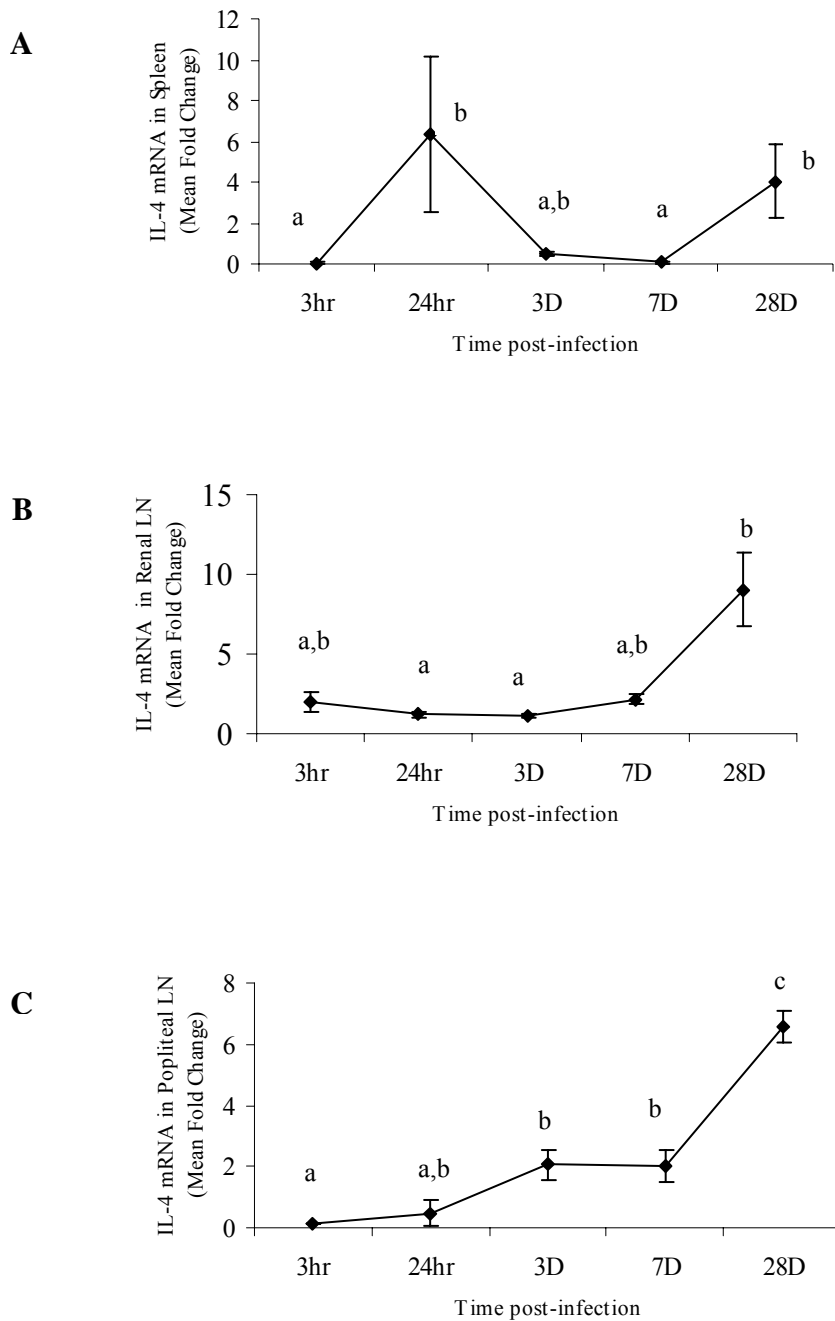


FIGURE 7. Quantitation of IL-4 mRNA in spleens (A), renal lymph nodes (B), and popliteal lymph nodes (C). Gerbils were necropsied at 3 hours, 24 hours, 3 days, 7 days, and 28 days after ID inoculation with *B. pahangi* L3. mRNA levels were measured by RT-PCR and values are expressed as mean fold change as compared with control animals. Superscript letters indicate statistical significance ($P < .05$). Values with the same letter are not statistically different.

Discussion and Conclusions

The focus of filariasis research on the well-established (chronic) stage of parasite infection has left a gap in the knowledge base of this disease. The events occurring during the first hours and days following filarid infection remain to be elucidated. Likewise, the significance of these early events on disease establishment and progression needs to be determined. Since it is impossible to determine the exact time of human host infection, laboratory animal models are uniquely suited to examining this question.

Some previous studies have examined the early time period of parasite migration. Ewert (1967) and Gordon and Crewe (1953) focused on transfer of the parasite from the vector to the host. Ash and Riley (1970) and Ah *et al.* (1973 and 1974) recorded *Brugia* larval recovery from gerbils before establishment of adult worms in the lymphatics. None of these studies, however, established a quantitative, controllable *in vivo* model to study the events of early larval migration within mammalian hosts. *Brugia* spp. infection in the permissive Mongolian gerbil closely resembles the common course of human infection, and the chronic disease state in this species has been well characterized (Jeffers *et al.* 1987, Klei *et al.* 1988, McVay *et al.* 1990). In addition, the ability of the gerbil to support all mammalian host life stages of *Brugia* allows its use as an *in vivo* model to document parasite migration, including both parasite location and migration time course.

The variety of locations in which larvae were recovered in our study substantiate the findings of Ah *et al.* (1974) that *B. pahangi* L3 have the ability to migrate through various connective tissues. Our studies further indicate that filarid larval migration through the skin to the lymphatic system occurs rapidly. At 3 hours after injection of *B. pahangi* L3 into the dermis of gerbils, the majority of the larvae remain in the immediate

vicinity of the infection site; however, by 24 hours after infection, large numbers of larvae are located significant distances away from the infection site (Table 2), at locations including peripheral (popliteal, subiliac) and central (renal) lymph nodes. These findings are compatible with the Ah and Thompson (1973) study that found larvae in regional lymphatics as early as 2.5 days after *Brugia* larval inoculation.

In addition to parasite migration, we found other important systemic events occurring soon after infection. A systemic inflammatory response, as determined by measurement of cytokine levels in the spleen and lymph nodes, was noted in infected gerbils within three hours of filarid infection. This response was comprised of elevations of the acute, pro-inflammatory cytokines TNF and IL-6. Both of these cytokines were elevated in all examined organs at 3 hours after infection and declined rapidly. The work of Babu and Nutman (2003) using human peripheral blood monocytes exposed to live *B. malayi* L3 has shown two important features of filarid larval migration. First, naïve T cell activation occurs within 24 hours. Our studies show that within this 24-hour period, significant intra-host parasite migration has occurred. Second, within 24 hours live *B. malayi* induce a predominantly Th1 cytokine response, with elevated levels of IFN γ , TNF, GM-CSF, IL-1 α , and IL-8. It is interesting to note that only exposure to live L3, not L3 antigen, affected cytokine expression suggesting physical contact of the larvae with host cells may play an important role in immune response. This leads to the question of the source of the cytokines present early in filarid infection. Though a definitive answer has not been determined, most work has focused on the role of monocytes (Raman *et al.*, Semnani *et al.* 2001, Babu *et al.* 2003), though both basophils and natural killer (NK) cells have been shown as a source of IL-4 in mice (Mitre *et al.*

2004, Balmer *et al.* 2002). Semnani *et al.* (2004) found that human Langerhans cells exposed to live L3 had down-regulated expression of IL-8 and antigen presentation genes and up-regulation of IL-18, a cytokine involved in Langerhans cell migration. These findings suggest that resident antigen presenting cells in the skin may play a role in establishing the cytokine environment present during early filarid migration.

Examination of the cell responses in mice infected intraperitoneal with *B. malayi* and *B. pahangi* also indicate an early host inflammatory response. In both immunologically intact mice and strains with adaptive immunity mutations, an influx of neutrophils is seen within 24 hours of infection and declines by 3 days (Rajan *et al.* 2002 and Ramalingam *et al.* 2003). Histologic examination of tissues from infected gerbils in our study shows a similar cellular response. Prominent foci of neutrophils were noted intradermally in infected animals within 3 hours after infection and were rarely noted by 3 days. Histology of tissues from control animals injected with larval wash media failed to exhibit these neutrophilic foci. These findings further support the presence of a pro-inflammatory host environment extremely early in filarid infection and also suggest a rapid decline in this inflammatory response. This decline in inflammation after early infection is consistent with hyporesponsive state characterizing the majority of human infections in endemic filariasis regions in which antigen-specific immune responses are down-regulated following initial infection (Nutman 1995). The cause of the down-regulation is unknown though it has been suggested that IL-10 and/or transforming growth factor (TGF) may play a role (Doetze *et al.* 2000, Levings *et al.* 2000, Levings *et al.* 2001). Studies in singly and multiply infected gerbils support a role for IL-10 in local regulation of the immune response (Chirgwin *et al.* 2004 a and b) since the highest IL-10

levels were measured in the primary site of infection (spermatic cord lymphatics) and the draining lymph nodes (renal lymph nodes).

Laboratory filarid infections are typically established by injecting the parasite into the subcutaneous tissues or the peritoneal cavity. In a natural infection, infective larvae migrate into the dermal layer of the skin through the puncture wound made in the epidermis by the vector; therefore, we utilized an intradermal infection technique to more accurately reflect natural larval transmission. Many models of cutaneous Leishmaniasis, another arthropod vector-borne disease, use intradermal inoculation of promastigotes to recreate natural transmission and have found differences with the traditional subcutaneous inoculations. Mitchell *et al.* (1981) found that lesions developed later in mice infected intradermally. Belkaid *et al.* (2002) found that CD8 (+) T cell deficient C57BL/6 mice inoculated intradermally with a low parasite dose could not control *Leishmania major* infection as compared to mice infected subcutaneously. These findings reinforce the importance of natural infection parameters in the development of parasitic infections.

Another interesting finding with Leishmaniasis has been the importance of the arthropod vector saliva on host response to infection. Studies in which sandfly salivary gland lysate has been coinoculated with *L. major* promastigotes have found that the vector saliva promotes a host Th2 response (Mbow *et al.* 1998), modulates host macrophage function (Hall *et al.* 1995), and can act to enhance infectivity (Titus *et al.* 1988). In our study the infective *B. pahangi* larvae were washed prior to inoculation to isolate the effect of the larvae on the host response; however, coinoculating L3 with mosquito salivary gland lysate would provide an interesting opportunity to examine the

possible vector contribution to infection. Given the potential role of vector components in disease development, effects of previous vector exposure must also be considered. Belkaid *et al.* (1998) found that upon coinoculation of *L. major* and salivary gland lysate, mice pre-exposed to vector saliva failed to develop the dramatically enhanced lesions and Th2 cytokine response seen in naïve mice. In addition, vaccination against the maxadilan molecule of sandfly saliva has been shown to protect mice against *L. major* infection (Morris *et al.* 2001).

Individuals in endemic filariasis regions are constantly exposed to bites from vector mosquitoes, both infected and non-infected and so the average human in these areas is likely to have multiple parasite exposures. We have found that Th2 polarization, as determined by increased IL-4 cytokine levels, occurs later in infection (after 7 days p.i.) and the Th1 cytokine response dominates the early period after infection. This response was only measured, however, in naïve animals. It is possible that previously exposed individuals may exhibit a different cytokine response over time. Studies of the immune response of gerbils to multiple *B. pahangi* infections, however, show that cytokine profiles of multiply infected gerbils did not differ significantly from those of gerbils with primary *B. pahangi* infections (Chirgwin *et al.* 2004 a and b). As in our study, Chirgwin *et al.* found an increase in IL-4 with time over the course of infection with the highest levels seen in chronically infected animals (>130 days post infection).

The Th1/Th2 polarization question is further complicated by the fact that individuals in endemic areas are likely exposed to multiple life cycle stages simultaneously. For example, there is evidence that host immune response differs depending on the parasite life cycle stage. Adult and microfilarial *B. malayi* stimulate

different antibody subclass patterns, with adult worms causing a rapid Th2 polarization (Lawrence *et al.* 1994). Since most work has been conducted on chronic infections, the role of L3 in the immune response is only recently gaining attention. Our study and the work of Babu and Nutman (2003) show a rapid and clear Th1 polarization. Devaney and Osborne (2000), however, found a rise in IL-4 transcription within 24 hours of infection of BALB/c mice with *B. pahangi* with no other cytokines found. The differences in these findings may represent the difference in response of a permissive (gerbil, human) and non-permissive (mouse) host.

With the presence of a Th1 proinflammatory environment during very early L3 migration, the role of inflammation in disease establishment and progression must be examined to determine if host inflammatory response acts in favor of the host or the parasite. In filariasis endemic regions, the segment of the population termed “endemic normal” (or putatively immune) are parasite antigen-free and microfilaremia-free, even though exposure is assumed to have taken place. These individuals are considered to have possibly developed a protective immunity to infection. Work with *Wuchereria bancrofti* (Dimock *et al.* 1996) has shown that antigen and microfilaria negative individuals demonstrate a Th1-like response with elevated IL-2 and IFN γ production and PBMC proliferation responses. These data suggest the proinflammatory response assists in development of immunity to infection.

Another hypothesis suggests that the production of Th1 cytokines and enhanced filarial specific T-cell activity observed during the filarial prepatent period in susceptible hosts actually assists growth and development of the larvae (Ravindran *et al.* 2001). This theory is supported by lack of such a TH1 cytokine environment in resistant hosts

(mice). The work of Osborne and Devaney (1999) showed that BALB/c mice infected with *B. pahangi* L3 experienced production of the anti-inflammatory cytokine IL-10 and Th2 polarization (IL-4 production) within 12 days after larval inoculation. Also, Babu *et al.* found that NK cells, producers of TH1 cytokines, are required to support growth of *B. malayi* in SCID mice. Though our study provides no direct evidence for either of these theories, the findings support inflammation as helping rather than hindering migrating filarial parasites. First, an inflammatory environment is established rapidly after larval inoculation, however larvae survive to migrate into lymphatics and establish infection, as indicated by worm recoveries at 28 days comparable to those seen in chronic gerbil filariasis studies (Klei *et al.* 1988, Klei *et al.* 1990). Second, histologically visualized larvae appeared non-degenerate at all time points even when inflammatory cells were present directly around larvae.

It has been suggested that the inflammatory lesions seen in filariasis are mediated by lipopolysaccharide-type activity from *Wolbachia*, the endosymbiotic bacteria present in most filarid nematodes. Taylor *et al.*(2000) found that extracts of *B. malayi* stimulated production of IL-1 β , TNF, and nitric oxide (NO) from murine macrophages and that the active component of the parasite extract exhibits LPS-like characteristics. Saint Andre *et al.* (2002) found similar results when injecting extracts of *Onchocerca volvulus* into murine corneas. Since live parasites do not stimulate inflammatory responses in murine macrophages (Taylor *et al.* 2000), it is proposed that inflammation in the host is induced only upon death of the filarid parasite, allowing release of the *Wolbachia* bacteria and its' LPS-like effects. This hypothesis would argue against the theory of inflammatory –assisted filarid growth. These *Wolbachia* studies, however,

were performed only in non-permissive hosts (mice) and only with extracts of adult parasites and microfilariae, not L3. Since the Th1 inflammatory environment is present within 24 hours of exposure to filarid L3 (current study, Rajan *et al.* 2002, Ramalingam *et al.* 2003, Babu *et al.* 2003), and a large amount of parasite death does not appear to have occurred by this time (current study), it seems unlikely that larvae would have died in large enough numbers within 24 hours to result in *Wolbachia*/LPS release sufficient to stimulate significant inflammatory responses. In addition, Fenn and Baxter (2004) examined the relative number of *Wolbachia* in *B. malayi* L3, L4, adults, and circulating microfilaria. The highest numbers of *Wolbachia* were found in the L4 and adult stages, while L3 contained only 0.35-fold as many *Wolbachia* as adult females.

Taylor *et al.* (2001) propose that episodes of acute inflammation (acute filarial lymphangitis) seen in chronic filariasis are due to natural attrition of adult parasites, microfilariae turnover, and L3-L4 larvae that fail to complete development. Th1 inflammatory cytokines, however, were found to peak early then decline rapidly. This would not occur if *Wolbachia*/LPS were being released upon death of the parasite through natural attrition, which would likely remain at a relatively constant level over time (barring therapeutic intervention). Another explanation may be that the L3 stage is responsible for acute inflammatory stimulation and, therefore, recurring episodes of acute inflammation in endemic regions are due to constant re-infection and exposure to migrating L3.

In conclusion, filarid larval migration occurs rapidly and stimulates a host systemic inflammatory response within 3 hours of exposure to migrating larvae. This response is characterized by elevated Th1 cytokines (TNF and IL-6) and dermal

neutrophilic foci. The classically described Th2 polarized immune response occurs after 7 days post-infection. These results emphasize the importance of the first few days of infection in the progression of, and response to, the disease of filariasis. An important point to determine with filarid infection is if inflammation acts to assist or curtail parasite development. Though our results tend to support an assisting role for inflammation, more work is required to fully answer this question. The factors determining the character of the cytokine environment and regulation of the resulting inflammatory response are still unknown. Likewise, the role of vector components in modulating host response and the potential for differing host responses to different parasite life cycle stages provide interesting paths for future research in this system.

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