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Carmen Nasarre
Louisiana State University and Agricultural & Mechanical College

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INFLAMMATORY RESPONSES OF THE JIRD TO
BRUGIA PAHANGI: PARASITE STAGE SPECIFICITY
AND ROLE OF THE MACROPHAGE

A Dissertation

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Louisiana State University and
Agricultural and Mechanical College
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in

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and Parasitology

by

Carmen Nasarre
D.V.M., University of Zaragoza, Spain, 1990
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ABSTRACT

The host systemic and peritoneal inflammatory responses against different stages of *Brugia pahangi* were compared in intraperitoneally infected-jirds. Systemic inflammatory responses were assessed by measuring the pulmonary granulomatous response to *B. pahangi* antigen-coated beads (PGRN). Peritoneal inflammation was characterized by enumeration of different cell types in peritoneal exudates following infection. Further, the toxoplasmacidal activity and TNF-α-like production of peritoneal macrophages were also characterized.

Infection with L3, L4, male worms, female worms, microfilariae (MF) or dead adult worms induced a rapid PGRN. This response decreased to levels of controls in jirds inoculated with living parasites at the chronic time period, indicating that viable worms are needed to downmodulate the PGRN, but MF are not required. Large numbers of female worms induced greater downmodulation of the PGRN than low numbers, and adult worm infection resulted in greater downmodulation of the PGRN than MF inoculation. These observations suggest that parasite burden is important in filarial-induced PGRN decrease. The greatest peritoneal inflammatory response was found in infections that resulted in MF production, and this response did not correspond to the level of PGRN indicating that MF act as potent inflammatory stimulus when compartmentalization occurs.
Gamma radiation inhibited the development and decreased the survival of *B.pahangi* L3. This effect was inversely related to the radiation dose used. Downmodulation of the PGRN occurred in infections with normal L3 or L3 irradiated with 15 krads, but not in infections of L3 irradiated with more than 15 krads, supporting the importance of the adult stage in the PGRN downmodulation in absence of MF. The PGRN decrease occurred at the time period when the molt to adult worms had just occurred, suggesting that larval stages are also involved in the downregulation phenomenon.

Macrophages from jirds inoculated with male or female *B.pahangi* were activated to kill *Toxoplasma* at 15 DPI coinciding with the peak of PGRN. TNF production peaked at 56 DPI and decreased markedly at 135 DPI. Absence of toxoplasmacidal activity was found in macrophages after 56 DPI corresponding to PGRN decrease. These data suggest that filaria-specific hyporesponsiveness may be associated with a downmodulation of macrophage function.
CHAPTER 1. INTRODUCTION, LITERATURE REVIEW AND OBJECTIVES

Human lymphatic filariasis is a long-term, parasitic disease principally caused by the filariid nematodes *Wuchereria bancrofti* and *Brugia malayi*. *Brugia timori* is responsible for an additional small percentage of cases. The estimated prevalence of lymphatic filariasis is currently 120 million (WHO, 1992; Ottesen and Ramachandran, 1995). *Wuchereria bancrofti* exists in Asia, Africa, the South Pacific, as well as in coastal areas of Brazil and some Caribbean islands, such as Haiti. *Brugia malayi* occurs in Malaysia, the Philippines, and some foci in India. *Brugia timori* is limited to the Indonesian islands of Timor and Flores (Denham and McGreevy, 1977; Ottesen, 1984a).

*Brugia pahangi* is a closely related species that is found in domestic and wild carnivores in Southeast Asia. *B. pahangi* has been reported as a natural infection in a few people from South Kalimantan, Indonesia (Palmieri et al., 1985). However, the significance of this filarial species as a human parasite is still undetermined, since the diagnosis of these human cases was based on the staining characteristics of the microfilarial acid phosphatase activity and was not confirmed by detailed morphological features of adult parasites or by successful development of microfilariae to infective larvae in the mosquito. *B.
pahangi has been experimentally transmitted to humans (Edeson et al., 1960).

PARASITE LIFE CYCLE

In the definitive host, adult nematodes live in the lymphatic vessels of testis, epididymis, spermatic cord, legs, arms, breasts, and abdominal, pelvic and retroperitoneal regions as well as in associated regional lymph nodes. The mature female worms produce microfilariae (MF) that migrate to peripheral blood. When ingested by suitable mosquito intermediate hosts, MF develop to infective third stage larvae (L3). The L3 migrate from the mosquito mouthparts and enter the definitive host thru the wound produced by the vector. Once in the skin, the L3 migrate to the lymphatics and lymph nodes to molt to the fourth stage larvae (L4) which develops and finally molts again to become mature female and male worms (Taylor, 1960; Schacher, 1962b). The prepatent period is approximately 3 to 8 months depending on the parasite species. Thus, L3, L4, adults and MF interact with the host immune system at different times and locations in the body. Furthermore, each of these larval stages is biochemically, morphologically and antigenically diverse and each exists for different durations during the life cycle (Ottesen, 1979; Partono, 1987). Therefore, stage-specific responses may occur during filarial infection and may be in part responsible for the different clinical patterns of the disease (Maizels and Lawrence, 1991).
Adaptation of filarial parasites to accomplish optimal transmission has probably lead to the development of strategies for prolonged survival in the host. For instance, adult worms may survive up to 30 years in humans, releasing MF throughout this time into the blood stream (Nutman, 1995). The persistence of filarial parasites in their mammal hosts constitute an important hallmark of filarial infection.

CLINICOPATHOLOGICAL MANIFESTATIONS

Lymphatic filariasis is a spectral disease; infection results in different clinical outcomes or conditions, each of which has been correlated with diverse states of filarial specific immune responsiveness (Ottesen, 1984b; King and Nutman, 1991). In endemic regions, infected individuals can be classified in five different categories (Ottesen, 1980).

1. Microfilaremic asymptomatic patients have no apparent clinical manifestations, have circulating MF in peripheral blood and are considered immunologically hyporesponsive to the parasite. These individuals normally constitute the largest group of the infected population and represent an important reservoir for transmission of the parasite. Recently, the use of lymphatic imaging techniques has detected morphologic changes such as dilatation and/or tortuosity of the lymphatic vessels (Case et al., 1992; Witte et al., 1993) in some of these microfilaremic asymptomatic patients. Renal abnormalities have also been reported (Dreyer, 1992).
2. Acute filariasis patients are those patients with periodic onsets of acute lymphatic obstruction manifested as lymphangitis, lymphadenitis and transient lymphedema accompanied by systemic symptoms of fever and malaise. These acute episodes, also known as "filarial fevers", last for a few days and may recur at irregular intervals of as much as 1 year duration. These individuals may or may not be microfilaremic. When these onsets repeat with increasing frequency patients may develop persistent lymphedema and chronic disease. Unfortunately, this group of patients has not been studied in detail.

3. Chronic lymphatic pathology patients are those that have developed chronic lymphatic obstruction manifested as elephantiasis, hydrocele or/and chyluria. Hydrocele is the accumulation of lymph between the tunicas that surround the testes. Chyluria is the excretion of chyle in the urine. Generally, these patients show pronounced filarial specific immune responses and amicrofilaremia. However, this group can be clinically, parasitologically and immunologically heterogeneous. For instance, hydrocele patients are often microfilaremic when compared to elephantiasis individuals that rarely are (Lammie et al., 1993; Addis et al., 1995).

4. Tropical pulmonary eosinophilia occurs in a small percentage of filarial infected individuals and is characterized by allergic, asthma-like pulmonary symptoms, apparently unrelated to lymphatic pathology. These patients
are hypersensitive to filarial antigens, particularly those derived from MF and show elevated eosinophilia, high levels of serum IgE and amicrofilaremia. Treatment with diethylcarbamazine effectively resolves the symptoms. Inadequately or non-treated cases evolve to pulmonary, interstitial fibrosis. Although the pathogenesis is still obscure, this syndrome could be explained as a severe allergic sensitization to MF antigens, resulting in entrapment and clearance of MF in the lungs and other organs of the reticuloendothelial system such as spleen and lymph nodes (Ottesen and Nutman, 1992).

5. Endemic normals are individuals who are continuously exposed to infected mosquitoes, have circulating filarial-specific antibodies, but have no clinical signs or evidence of infection and are considered putatively immune. This group, however, is difficult to define. Strict clinical, parasitological and serological criteria must be applied in an endemic population to distinguish truly resistant individuals from those that harbor occult or low grade infections. Some detailed studies have reported the endemic normal as a rare individual (Freedman et al., 1989; Day, 1991). Furthermore, it has been hypothesized that protective resistance may result primarily from a dynamic process associated to continuous reinfection rather than to naturally acquired immunity (Day, 1991).
Although most of the patients fall into one of these categories, individuals with overlapping syndromes do exist. Chronic lymphatic pathology patients with microfilaremia have been identified. In these patients immunoregulatory mechanisms that operate are more difficult to explain (Lammie et al., 1993). Studies by Lammie et al. (1993) in the Haitian community of Leogane have revealed that many of the patients with hydrocele were microfilaremic (25 of 42), meanwhile only 1.5% of patients with chronic limb involvement had MF in peripheral blood. Microfilaremic individuals with hydrocele lacked antifilarial immune reactivity suggesting that pathogenesis of hydrocele formation may be initiated by mechanisms other than immune or inflammatory reactions. Data (Lammie et al., 1993; Addis et al., 1995) indicate that microfilaremic patients with hydrocele are in an earlier stage of the disease than amicrofilaremic hydrocele patients supporting the hypothesis that microfilaremic asymptomatic individuals progress to an amicrofilaremic immune responsive state (Bundy et al., 1991).

Interestingly, clinical signs of lymphatic filariasis appear more rapidly and are more severe when the infection occurs in individuals that have not been exposed previously to the parasite. This occurs in individuals that are not native to endemic areas. This phenomenon was first documented when thousands of American soldiers moved temporarily to the South Pacific area during World War II.
Approximately one third of soldiers exposed to filariasis developed acute onsets of lymphangitis, lymphadenitis or genital inflammation with absence of microfilaremia (Wartman, 1947; Ottesen, 1980; Partono, 1987). Symptoms remitted when affected soldiers left endemic areas. Similar responses occurred in Indonesians of the same ethnic background who moved from filariasis free areas to areas endemic for filariasis (Partono et al., 1978). When these affected individuals leave filariasis prevalent areas the clinical signs tend to resolve. However, if they remain continuously exposed to infective larvae, they develop a more rapid and severe form of elephantiasis (Wartman, 1947; Partono et al., 1978). Exposure to parasites when the immune system is not fully competent as may occur during pre- or neo-natal life may result in specific filarial immune tolerance with subsequent attenuation of disease manifestations (Nutman et al., 1986; Ottesen, 1989). Thus, differences in response among individuals of similar ethnic background can be partly explained in this manner.

The clinical manifestations of Bancroftian filariasis differ in some aspects with those of Brugian filariasis. In Bancroftian filariasis clinicopathologic changes are more extensive affecting the whole leg and arm, and also commonly occur in the male genitalia. During the acute period the involvement of male genitalia lymphatics leads to funiculitis, epididymitis and orchitis, manifested clinically
by pain, swelling and tenderness. Repeated, acute episodes of orchitis and epididymitis often result in hydrocele. Hydrocele is the commonest chronic manifestation of Bancroftian filariasis in many parts of the world (Partono, 1987). Microfilariae are sometimes present in the hydrocele fluid, even though they may be absent from peripheral blood. Some infected individuals develop chyluria, which is associated with blockage of the retroperitoneal lymph nodes below the cisterna chyli resulting in efflux and flow of the mesenteric lymphatics directly into the renal lymphatics. The urine of patients with chyluria contains large amounts of dietary lipids, proteins and possibly fat-soluble vitamins, leading to weight loss and debilitation. If severe and persistent, chyluria may also result in lymphopenia due to loss of lymphocytes in the urine (WHO, 1985).

In Brugian filariasis elephantiasis of the legs occurs below the knee and only occasionally affects the arms below the elbow. Genital involvement rarely occurs (Review in Denham and McGreevy, 1977).

PATHOGENESIS AND PATHOLOGY OF LYMPHATIC FILARIASIS

Experimental studies in laboratory animals and observations in natural human infections have helped in the understanding of lymphatic lesion pathogenesis caused by filarial parasites (Ewert et al., 1972; Gooneratne, 1973). After penetrating the skin, the L3 locate in afferent lymphatic vessels, and subcapsular and cortical sinuses of
lymph nodes where they molt twice. The presence of the parasites, their cast-off sheaths, and their molting fluids and other secretory/excretory products presumably initiate morphological changes in the vessel characterized by luminal dilatation or "lymphangiectasis", endothelial proliferation and thickening of the lymphatic wall with inflammatory cells and fibrosis (O'Connor, 1932). Adult parasites probably cause an important mechanical damage to the lymphatic vessel wall and valves especially due to their continuous movement leading to lymph vessel dilatation (Von Lichtenberg, 1987). More severe lymphatic damage occurs when worms induce granulomatous reactions resulting in partial or complete occlusion of the vascular lumina. Granulomas are most typically associated to the presence of dead or dying adult worms. However, MF and other parasite products are probably involved in this inflammatory process as well (Meyers et al., 1976; Klei et al., 1982; Von Lichtenberg, 1987). Granulomas often surround parasites and are characterized by accumulation of macrophages, eosinophils, lymphocytes, plasma cells, and multinucleated giant cells with fibrous tissue proliferation developing in the periphery. Other times, more exudative reactions occur and consist of areas of necrosis with polymorphonuclear leukocytes, especially eosinophil, but also neutrophil infiltration surrounding the worms or worm remnants (O'Connor and Hulse, 1935; Von Lichtenberg, 1987). Lymphatic vessel dilatation with no inflammation may be the
only histopathologic change accompanying the worms. Sometimes a mild non-granulomatous, parietal infiltrate may be present in the lymphatic vessel walls (Jungmann et al., 1991). These slight changes may reflect a suppression of filarial specific immune reactivity. The damage of valves, along with the lymphatic dilatation, lymph stasis and lymph vessel thrombosis by granulomas result in increased hydrostatic pressure and increased permeability of the walls to the lymph. Consequently, there is leakage of fluid with a high concentration of protein into the adjacent tissues. Protein-rich lymph in the tissues is a potent stimulator of fibrogenesis and neovascularization. These latter processes are also favored by the inflammatory reaction that the worms and their products induce. The mechanism of fibrosis in filariasis is not well understood, but is probably associated with both mechanical blockade of lymph flow and parasite-induced inflammation. Surgical ligation of lymphatic ducts in rabbits (Wolfe et al., 1983) and dogs (Snowden and Hammerberg, 1989) induced mild lymphedema with little fibrotic changes indicating that fibrosis in elephantiasis patients is a more complex process.

Several authors have proposed that hypersensitivity reactions mounted against parasites and their products are important factors leading to severe inflammation (Rifkin and Thompson, 1945; Lichtenberg, 1957). Thrombolympangitis and thrombophlebitis with parasite material diffusing into the
interstitium and exacerbating the inflammatory process have been interpreted as a phenomenon generically related to the Arthus reaction (Lichtenberg, 1957). Further, increased circulating immune complexes and enhanced complement consumption have been found in individuals with elephantiasis when compared with asymptomatic microfilaremic patients (Gajanana et al., 1982; Prasad et al., 1983; Nath et al., 1991).

Tissues affected with lymphedema are likely to be more prone to bacterial infections, since the normal texture of the skin covering affected locations may be altered and the underlying compromised lymph drainage cannot effectively remove incoming pathogens. Bacterial complications lead to more severe pathology and have been reported to form part of many filarial cases (Olszewski et al., 1990; G.Dreyer in Maizels et al., 1995).

Finally, it should be pointed out that the scarcity of autopsies in human filariasis patients has greatly hampered the understanding of lesion development in this disease.

**IMMUNE RESPONSES IN HUMAN LYMPHATIC FILARIASIS**

The different clinical outcomes of filarial infection are presumably related to the capacity of the host immune system to respond to filarial antigens (Ottesen, 1980; King and Nutman, 1991). Asymptomatic microfilaremic individuals have impaired immune responses to filarial antigens when compared with the strong immune responsiveness of individuals
with lymphatic pathology or tropical pulmonary eosinophilia, who are usually amicrofilaremic (Ottesen et al., 1977). Low lymphocyte blastogenic responses to filarial antigen have been consistently found in microfilaremic asymptomatic individuals in contrast with the remarkably high lymphocyte proliferative responses of individuals showing pathology and amicrofilaremia (Ottesen et al., 1977; Piessens et al., 1980; Nutman et al., 1987a; King et al., 1992). Antifilarial antibody levels also differ between microfilaremic and amicrofilaremic chronic filariasis patients. Hyporesponsive microfilaremic asymptomatic individuals have low levels of IgG1, IgG2 and IgG3 (Nutman et al., 1987b; Ottesen et al., 1982) but have high levels of IgG4 (Ottesen et al., 1985; Hussain et al., 1987). Conversely, amicrofilaremic pathology patients produce high levels of IgG1, IgG2 and IgG3 and little or no IgG4 (Hussain et al., 1987; Maizels et al., 1995). In microfilaremic asymptomatic patients, antifilarial IgG4 response correlates with microfilaria counts (Kwan-Lim et al., 1990) and levels of this isotype drop markedly after treatment with the microfilaricidal drug diethylcarbamazine (Maizels et al., 1995; Sartono et al., 1995). The IgG4 subclass has been demonstrated to possess blocking activities that would prevent IgE-mediated hypersensitivity reactions (Aalberse et al., 1983).

As may be expected, important differences are also observed in cytokine profiles between defined patient
populations. Peripheral lymphocytes of microfilaremic asymptomatic patients produce high levels of IL-4 and IL-5 in response to filarial antigens but make low levels of IFN-γ, IL-2 and GM-CSF. In contrast, lymphocytes of symptomatic amicrofilaremic individuals respond with increased levels of IFN-γ and IL-2 production (Nutman et al., 1987a; King et al., 1993). IL-10 has been observed to be elevated in asymptomatic microfilaremic patients compared to chronic pathology patients, and most importantly, neutralizing antibodies against IL-10 and TGF-beta restore the in vitro hypo-responsiveness to parasite antigens present in asymptomatic microfilaremic patients, (King et al., 1993). However, others investigators have been unable to restore unresponsiveness of cultured T cells by adding anti-IL-10 (Maizels et al., 1995). Treatment with diethylcarbamazine (DEC) and ivermectin restores immune responsiveness in asymptomatic microfilaremic patients (Piessens et al., 1981; Lammie et al., 1992). Interestingly, after elephantiasis patients are treated with DEC anti-filarial immune responsiveness is enhanced suggesting that all filarial infections, not necessarily including microfilaremia, are able to induce some degree of immunodepression (Maizels et al., 1995). Other studies have also found that some asymptomatic amicrofilaremics and some elephantiasis patients manifest specific hyporesponsiveness presumably due to active infection (Yazdanbakhsh et al., 1993). King et al. (1993)
believe that modulatory cytokines may be responsible for the up or down regulation of the immune responses in filarial infected individuals, by responding either with Th1 or Th2 cytokine profiles, respectively. Based on this hypothesis, pathology would manifest when Th1 cytokines predominate, whereas the appearance of a Th2 phenotype would lead to a hyporesponsive state typical of microfilaremic asymptomatic individuals. Conversely, Maizels et al. (1995) argue that high T-cell responsiveness, release of Th1 cytokines and IgE production may not be implicated in the development of elephantiasis. These authors have found that some elephantiasis patients exhibit a microfilaremic-like phenotype, with high IgG4 levels and hyporesponsive T cells. Furthermore, long-term treatment with diethylcarbamazine reduces symptoms (Partono et al., 1984) and results in restoration of T cell hyperresponsiveness and release of cytokines, such as IFN-γ, contradicting again that these immune factors lead to pathology (Maizels et al., 1995; Sartono et al., 1995). Moreover, many endemic normals have levels of IgE and T-cell responses similar to elephantiasis patients without showing any clinical signs. However, it may be important to note that levels of IgG1, IgG2 and IgG3 in endemic normals are always lower than in chronic pathology patients (Maizels et al., 1995). These IgG isotypes, especially IgG3, are good at fixing complement and may participate in immune complex reactions. Deposition of
immune complexes in filariasis has not been closely investigated, but it has been proposed by some authors as a relevant factor in pathology (Lichtenberg, 1957; Klei et al., 1982; Hussain et al., 1987; Nath et al., 1991).

It has been suggested that endemic normals are able to kill incoming L3 preventing establishment of worms in the lymphatic system and initiation of pathology (Maizels and Lawrence, 1991). One immunological study in an endemic area in the Pacific revealed that filarial exposed non-infected children are more responsive than the equivalent group of adults, suggesting that the so called "endemic normals" may become specifically desensitized with continuous exposure eventually being able to progress to the microfilaremic hyporesponsive state (Ottesen et al., 1977). Long-term studies in this filariasis group would help enormously in the understanding of the immune response dynamics and risk factors for development of pathology or immune tolerance.

Pre- or peri-natal exposure to filarial antigens has been proposed to be partly responsible for the state of immune tolerance in filariasis (Weil et al., 1983). Anti-filarial IgM (Dissanayake and de Silva, 1980) and IgE (Weil et al., 1983) have been found in the cord blood of human fetuses and microfilariae have occasionally been found in the bloodstream of newborns (Loke, 1982). Moreover, children born from microfilaremic mothers are more prone to become microfilaremic than children born from amicrofilaremic
mothers (Lammie et al., 1991). Studies in Brugia-infected jirds have demonstrated that offspring born from infected mothers have higher levels of microfilaremia and develop less severe pathology than the progeny from uninfected jirds (Klei et al., 1986).

Monocytes (Piessens et al., 1980), serum suppressor factors (Piessens et al., 1980) or suppressor CD8+ T lymphocytes (Piessens et al., 1982) have been reported as possible active suppressors in studies with Brugian filariasis patients. However, other similar studies in Bancroftian filariasis patients have failed to demonstrate such suppressor cell populations (Nutman, et al. 1987a).

Few studies have addressed host genetic influences on the immune responses and outcomes of filarial disease. Ottesen et al. (1981) observed familial clustering of infection in studies performed in Polynesia but failed to find any linkage to HLA-A or B-locus specificities. An association between chronic lymphatic pathology and HLA-B15 has been found in patients from areas of South India and Sri Lanka where filariasis is endemic (Chan et al., 1984). More recently, M. Yazdanbakhsh et al. (in Maizels et al., 1995) have found that one class II allele is correlated with progression to elephantiasis. In addition, these studies have revealed that asymptomatic microfilaremic individuals have higher frequency of 2B3 (epitope present on DQW6, 8 and 9) than lymphatic pathology patients (Maizels et al., 1995).
EXPERIMENTAL ANIMAL MODELS

Advances in our understanding of filarial pathogens is highly dependent on animal models, especially because of the parasite longevity, the difficulty to determine parasite burdens in vivo, the inavailability of autopsy specimens and the impossibility of performing longitudinal studies, since treatment to clear infection is readily available and effective. Therefore, patients diagnosed with filariasis must be treated, making it impossible to investigate the evolution of the diverse clinical conditions. The main constraint in filariasis research is the lack of an adequate experimental model which is susceptible to infection and at the same time is well defined immunologically. The only known animal in which *Wuchereria bancrofti* develops to patency is the silvered leaf monkey, *Presbytis cristatus* (Palmieri et al., 1980). The difficult availability of this monkey species makes its use as an animal model not possible. A variety of animals are permissive for *B. malayi* and *pahangi*. These include, the cat (Denham and Fletcher, 1987), dog (Schacher and Sahyoun, 1967), mongolian jird (Ash and Riley, 1970a), immunodeficient mice (Suswillo et al., 1980) and ferret (Crandall et al., 1982), which have been used for the study of filariasis. Other animal models, such as some rat strains (Cruickshank et al., 1983), the golden hamster (Ash and Riley, 1970b; Malone et al., 1974), the guinea pig (Ahmed, 1967), and the rabbit (Ahmed, 1967) have less
consistent permissiveness and excessive variation in susceptibility to Brugia infection. Therefore, their use has been limited. Immunocompetent mice do not support the development of Brugia spp. to patency (Chong and Wong, 1967; Howells et al., 1983). However, they allow development of early larval stages before reaching maturity and short-term survival of inoculated microfilariae (Grove et al., 1979). The inoculation of different life cycle stages in a nonpermissive model, although informative, may not totally represent the real situation. The jird is probably a more suitable model to investigate the role that each of the life cycle stages plays in the induction and downregulation of immune and inflammatory responses during filarial disease. In this model, the time periods at which the life cycle stages molt and the adults release MF have been accurately defined. Furthermore, studies have demonstrated that intraperitoneal and subcutaneous infections result in similar lesions (Jeffers et al., 1987).

Cat

Cats and dogs are natural hosts for B. pahangi and B. malayi in areas of Southeast Asia. Filarial infection in cats closely resembles that caused by Wuchereria and Brugia in humans, and provides important clues for the understanding of the disease dynamics and immunity (Grenfell et al., 1991). In primary infections the adult worms that survive and establish successfully in the lymphatic system are long
lived, and microfilaremias remain stable in most cats (Denham and Fletcher, 1987). Although, patterns of worm recovery first suggested the development of concomitant immunity against incoming L3 (Grenfell et al., 1991), more recent parasitological observations contradict this interpretation. Interestingly, amicrofilaremic cats appear to be able to also kill adult parasites (Denham et al., 1993). A small proportion (up to 15%) of cats with primary infections and a larger proportion (up to 40%) of cats with repeated infections clear their microfilaremias (Denham, 1983; Fletcher, 1986). Those cats with repeated infections often manifest a transient, recurrent lymphedema (Denham and Rogers, 1975). The clearance of microfilaremia in this animal model coincides with the peak production of anti-microfilarial antibodies (Ponnudurai et al., 1974) mimicking a similar response in human patients (Grenfell et al., 1991; Maizels and Lawrence, 1991). In conclusion, the cat-\textit{B. pahangi} model supports the hypothesis that the development of pathology is related to the amicrofilaremic state and the appearance of immunity. However, in spite of the apparent similarity in disease dynamics, cats do not develop the chronic lymphatic pathology seen in human lymphatic filariasis (Denham et al., 1994).

**Dog**

The dog is a natural host for \textit{B. pahangi} and a good model to study lymphatic filariasis. Infected dogs develop
a variety of clinical changes that parallel the human filariasis spectrum (Snowden and Hammerberg, 1989). Pathologic changes in the dog appear to be more severe and of earlier manifestation than in the cat (Schacher and Sahyoun, 1967; Schacher et al., 1969). Furthermore, transient limb lymphedema and lymphadenopathy is not necessarily associated with multiple infections (Snowden and Hammerberg, 1989).

**Ferret**

Ferrets are particularly hyperresponsive to infection and develop a syndrome more characteristic of hyperresponsive patients manifesting TPE or filarial fevers, or those non endemic populations that are primarily exposed (Hines et al., 1989). Subcutaneous infection of ferrets with *B. malayi* results in establishment of adult worms in the lymphatic system and production of microfilariae after approximately three months. Most ferrets become amicrofilaremic before eight months postinfection and only a small percentage (10 to 15%) develop prolonged microfilaremia. Clearance of microfilaremia is associated with eosinophilia, prominent immunoresponsiveness to filarial antigens and widespread multifocal granulomas. These granulomas occur principally in liver, lung, and lymph nodes and are characterized by an inflammatory infiltrate of primarily macrophages and eosinophils with occasional entrapped dead MF and a typical Splendore-Hoepli material that closely resembles the Meyers-Kourewnaar bodies described in TPE lesions of humans.
(Crandall et al., 1982; Crandall et al., 1984; Hines et al., 1989). Following a single infection lymphedema of the legs is only occasionally apparent and always transient. When ferrets receive multiple infections, those that become amicrofilaremic often develop sustained lymphedema of the legs and do not become patent on reinfection (Crandall et al., 1987). Lymphedema has been observed to persist in some animals for more than three years. Inoculations of microfilariae induce partial resistance to challenge infection and promotes development of lymphatic disease coinciding with the clearance of microfilariae (Crandall et al., 1990). Thus, acquired resistance in the ferret appears to be associated with an amicrofilaremic state. The lymphangitis and lymphatic dilatation that occurs in ferrets is similar to that observed in other experimental hosts and humans (Hines et al., 1989). The ferret model may be useful in studies to clarify the relationship between acquired resistance and development of pathology as well as the implication of certain parasite stages, such as MF, in disease and immunity.

Mouse

The murine model is particularly advantageous to study specific cellular and humoral responses during filarial infection because of the availability of immunological reagents. Although immunologically intact mice are not fully permissive for Brugia spp. infection; depending on the route
of inoculation and the life cycle stages used, parasites may survive long enough to be studied immunologically (Lok and Abraham, 1992). In contrast, infective larvae develop to full maturity in athymic nude mice (Suswillo et al., 1980) and in scid mice (Nelson et al., 1991). However, reconstitution of immunodeficient mice with T cells confers the capacity to mount effective humoral and cellular responses to the parasite (Vickery et al., 1983; Vickery and Vincent, 1984) which indicates that T cells are important in resistance. The immunodeficient murine model has also provided important insights regarding lesion pathogenesis. The use of the immunodeficient mice has revealed the existence of two possible mechanisms of disease; one is independent of the immune response and the result of the presence of the parasites in the lymphatics of immunodeficient mice, where they induce endothelial cell proliferation, severe lymphatic vessel dilatation and finally lymphedema and elephantiasis (Vincent et al., 1984; Nelson et al., 1991). The second is dependent on the immune response and occurs when immunodeficient mice are reconstituted with cells from immunocompetent mice sensitized with filarial antigens resulting in pronounced inflammatory responses around the parasites that leads to lymphatic damage and obstruction, lymphedema and elephantiasis (Vickery et al., 1991). Studies in mice have contributed information on the types of immune responses mounted against filarial parasites.
Several studies (Bancroft et al., 1993; Pearlman et al., 1993a; Lawrence et al., 1994) have shown that these immunocompetent animals develop local and systemic dominant Th2 responses after inoculation of Brugia MF, MF extracts, irradiated L3 or adult worms. Acquired resistance to MF inoculated intravenously or intraperitoneally occurs after subcutaneous sensitization with MF antigens and correlates with a Th2 response (Pearlman et al., 1993b). Resistance to L3 challenge is accomplished after vaccination with irradiated L3, and this again is associated with a Th2 response (Hayashi et al., 1989; Bancroft et al., 1993). Intraperitoneal inoculation of adult female or male worms induce a rapid Th2 response, whereas inoculation of MF results in an early Th1 phenotype (Lawrence et al., 1994). Interestingly, studies in IL-4 deficient mice inoculated intraperitoneally with different stages of B. malayi indicate that the survival of adult worms, MF or L3 appears not to be affected by a Th2 response (Lawrence et al., 1995). The role of IL-13, however, has not been investigated.

Jird

Jirds support complete development of both B. malayi and B. pahangi (Ash and Riley, 1970a; Ash and Riley, 1970b). However, B. pahangi has been used more extensively than B. malayi for experimental infections in the jird because the prepatent period is shorter, the microfilaremia is of higher levels and the location of adult worms resembles more closely
that reported for natural hosts (Ash, 1973). Furthermore, no significant pathological or immunological differences were observed in a comparative study of jirds infected with B. malayi or B. pahangi (McVay et al., 1990). In jirds, subcutaneous inoculation of L3 in the inguinal area results in localization of the parasites in lumbar, spermatic cord and testicular lymphatics, and associated lymph nodes. Pathological findings resemble those reported in humans and other experimental animals (Ah and Thompsom, 1973; Vincent et al., 1980; Connor et al., 1986; Klei et al., 1986). The pathological changes in the jird lymphatics are characterized by lymphatic vessel dilatation, inflammatory and fibrous thickening of the vascular wall and typical intralymphatic granulomatous thrombi (Ah and Thompsom, 1973). However, lymphedema or elephantiasis, as seen in human lymphatic filariasis, are not observed in the jird. First evidence of lymphatic inflammation appears to coincide with the final molt of worms to become adults (Vincent et al., 1980). The kinetics of the inflammatory response in the lymphatic system and the granulomatous reactivity around Brugia antigen-coated beads embolized to the lungs have demonstrated the presence of several states in the jird-B. pahangi model that mimic some of the conditions discussed in human filariasis. These include, the acute responsive state, the microfilaremic hyporesponsive state, a hyperresponsive state induced by antigen immunization, and an immune state induced by
vaccination (Klei et al., 1981; Weil et al., 1983; Yates and Higashi, 1985; Kazura et al., 1986). Numbers of lymphatic granulomatous lesions are greatest between 60 and 90 days postinfection (Vincent et al., 1980), decreasing significantly thereafter (Klei et al., 1988; Klei et al., 1990). The antigen reactivity in the lungs measured by granuloma areas around antigen-coated beads (PGRN) is maximum at 14 DPI (Rao et al., 1995). This period of peak PGRN coincides with the maturation of larval stages to become early adult worms. PGRN decreases markedly as the infection progresses to the chronic period (Klei et al., 1981). This downregulation occurs before adult worm maturation and patency, and is associated with the presence of mature adult worms within lymphatics and with the onset and persistence of MF. Blastogenic responses of renal lymph node cells peak early in infection and decrease thereafter (Rao et al., 1995). Kinetics of axillary lymph node cell proliferation is strikingly different from that of renal lymph node cells draining the site of infection in that no downmodulation is observed in the former. Proliferative responses of spleen cells peak later than those of renal lymph node cells, and decrease similarly in the chronic state of infection coinciding with persistent microfilaremia (Lammie and Katz, 1983; Leiva and Lammie, 1989; Klei et al., 1990). This decreased splenic responsiveness has been shown to be associated with the presence of an adherent cell population
in the spleen (Lammie and Katz, 1984). Filarial-specific antibody levels increase gradually during infection and remain elevated throughout the chronic state (Klei et al., 1990). Peripheral blood eosinophils peak during the acute period coinciding with the molt to the adult stages, and decrease significantly during the chronic period (Klei et al., 1990; McVay et al., 1990). Converse to what is seen in ferrets and cats, multiple challenge infections do not induce protection in microfilaremic jirds, and do not significantly modify preexisting lymphatic lesions or reactivity around antigen-coated beads in the lungs (Klei et al., 1988). Likewise, chronically infected, amicrofilaremic jirds are susceptible to reinfection and challenge infection does not increase lymphatic pathology (Lin et al., 1995). Protective resistance can be achieved in jirds by vaccination with irradiated L3 (Yates and Higashi, 1985; Chusattayanond and Denham, 1986; Petit et al., 1993). Interestingly, vaccinated jirds manifest increased lymphatic pathology and higher PGRN after challenge when compared to normal L3 infected or uninfected animals (Petit et al., 1993). Preexistent intraperitoneal infections, however, result in decreased lymphatic pathology after subcutaneous challenge (Klei et al., 1987).

**THE ROLE OF THE MACROPHAGE IN LYMPHATIC FILARIAsis**

The macrophage (MAC) is implicated in the pathogenesis of lymphatic filariasis primarily because this cell is a
principal component of the granulomatous inflammation that occurs in lymphatic and TPE lesions. As an essential arm and key cell of the immune response, the MAC may also play an important effector and regulating role in the immune response directed towards filariae. The different filarial life cycle stages have numerous opportunities to interact with the MAC in diverse compartments of the monocyte phagocytic system; L3 in the skin and lymph nodes, MF in the blood, spleen, lymph nodes and granulomas, adults and possibly L4 in granulomas and lymph nodes. At these sites, the MAC performs its complex variety of functions together with other immune effector cells.

As L3 penetrate the mammalian host, the MAC of the skin may function as antigen presenting cell (APC) and hypothetically may be able to direct infection into different outcomes depending on genetic background of the host (eg: Major histocompatibility complex, natural immunity, etc.) The expression of surface molecules (eg: Mac-2, Receptor for C3bi, Fc receptor) vary in MACs from different tissues (Nibbering et al., 1985; Nibbering et al., 1987) which could partly influence the presumed differences in host responses to each of the filarial life cycle stages. Thus, parasite-MAC interactions may be crucial throughout the infection and upon continuous reinfections driving the cascade of immune responses that develop. Filarial parasites are often found covered by numerous inflammatory cells. The MAC is the most
abundant (Soulsby, 1963; Jeska, 1969; Jeffers et al., 1987). MACs have Fc and complement receptors (Lay and Nussenzweig, 1968) that mediate adherence to the parasite surface and may be important in a larvacidal mechanism. Rat MACs have been implicated in cytotoxicity reactions against *B. pahangi* L3 (Chandrashekar et al., 1985) and *B. malayi* MF (Chandrashekar et al., 1985; Chandrashekar et al., 1986) in vitro.

MACs are known to be important producers of cytokines and other molecules that function as mediators of immune and inflammatory responses (Review in Nathan, 1987; Rappolee, 1992). These products may have pro- or anti-inflammatory effects. The products that promote inflammation include TNF-α, IL-1, IL-6, colony-stimulating factors, complement components, coagulation factors, arachidonic acid metabolites, platelet-activating factor, platelet-growth factor, and fragments of fibronectin and elastin. The ultimate objective of inflammation is destruction of the pathogen. In order to fulfill this task the MAC must be activated. Adams and Hamilton (1988b) redefine the concept of MAC activation as competence to mediate or complete a complex function such as processing and presentation of antigen or killing of microorganisms. They distinguish activation from capacities or attributes that can be measured immunologically or biochemically such as number or affinity of a certain receptors or secretion of a particular molecules (Adams and Hamilton, 1988a).
More than one signal is required to fully activate MACs. In the murine model, a priming signal, IFN-γ and a triggering signal, TNF-α or LPS are necessary (Adams and Hamilton, 1987). Signals that suppress MAC activation are less studied but include TGF-β (Tsunawaki et al., 1988), IL-4 (Liew et al., 1989; Gautam et al., 1992), IL-10 (Mosmann and Moore, 1991; Gazzinelli et al., 1992), prostaglandins (review in Schultz, 1991) and α₂-macroglobulin-protease complexes (Adams and Hamilton, 1988a; Crawford et al., 1994). The two main mechanisms that mediate parasite killing are the L-arginine-dependent nitric oxide intermediate (NOI) and the reactive oxygen intermediate (ROI) pathways. Mouse MACs stimulated with IFN-γ and TNF secrete large amounts of NO (Ding et al., 1988). NO production seems to be the principal effector mechanism in many murine systems of MAC-mediated microbial killing (Green et al., 1991). However, few studies have been reported on the role of NO in cell-mediated helminth killing. In the Schistosoma-mouse model, activated MACs kill schistosomula thru the production of NO (James and Glaven, 1989). On the other hand, ROI released by phagocytes stimulated thru Fc receptors do not affect schistosomula viability (Scott et al., 1985). In some systems, however, ROI has been proved to be important. Filarial parasites are known to be highly sensitive to ROI. Particularly, hydrogen peroxide has been found to be the most toxic oxygen species for the filaria Onchocerca cervicalis (Callahan et al., 1990).
Schistosomula are killed by murine (James and Glaven, 1989) and human (Cottrell et al., 1989) MACs previously activated by lymphokines and resistance in the mouse is associated with production of Th1 cytokines, particularly IFN-γ (Smythies et al., 1992). Th1 and Th2 subsets are characterized by the secretion of a contrasting and cross-inhibitory array of cytokines. Th1 cells produce IFN-γ, IL-2 and TNF-β which are proinflammatory cytokines and promote MAC activation. Th2 secrete IL-10, IL-4 and IL-5 which are involved in B cell and eosinophil development, antibody production and MAC deactivation (Mosmann and Coffman, 1989).

Interactions of MACs and eosinophils appear to be important in parasitic and allergic conditions. MACs release eosinophil chemotactic and activating factors (Burke et al., 1991). MACs secrete eosinophil cytotoxicity-enhancing factor which primes eosinophils for enhanced cytotoxicity against schistosomules (Elsas et al., 1987; Lenzi et al., 1985). Conversely, several studies (Haque et al., 1982; Nogueira et al., 1982; Chandrashekar et al., 1986) have found that eosinophil granules and other products are chemotactic for MACs and enhance MAC cytotoxicity for nematode larvae. Although the mechanisms of action of DEC are still uncertain, DEC-mediated clearance of MF is thought to be dependent on humoral and/or cellular factors. Several studies have implicated the MAC in the destruction of MF during DEC.
treatment and it has been proposed that initial DEC damage of MF may trigger MAC-mediated cytotoxic mechanisms (Hawking et al., 1950; Hayashi et al., 1983).

MACs can release many cytokines that stimulate fibroblasts and promote fibrosis, such as platelet-derived growth factor (PDGF), TNF-α, insulin-like growth factor-1 (IGF-1), TGF-β, fibroblast growth factor (FGF), colony stimulating factor 1 (CSF-1), IL-1-β and fibronectin (Review in Kovacs, 1991). Although the major mechanisms responsible for the exuberant fibrous tissue deposition observed in some elephantiasis patients are uncertain, MAC products are probably involved, especially in promoting the varying degrees of fibrosis that generally ensues granulomatous inflammation.

The role that the MAC may play in lesion initiation, persistence or termination during filarial infection may be of central importance to understand disease pathogenesis. More work is needed to clarify the relevance of the MAC to immune and inflammatory responses in filariasis.

THE IMPACT OF DIFFERENT LIFE CYCLE STAGES ON THE IMMUNE AND INFLAMMATORY RESPONSE

The different filarial developmental stages are morphologically, antigenically and biochemically different. They are unlikely to induce an identical immune response in the definitive host. The ability of an individual’s immune system to cope with each of the life cycle stages may partly
contribute to the different outcomes of infection. It is also possible that each stage of the life cycle may have an effect on the immune response to subsequent stages or infections.

MF have been implicated as a likely candidate to induce immunosuppression in filariasis. Evidence for this exists in both humans and experimental animal models. Microfilarial extracts from *B. malayi* have been found to suppress mitogen-driven lymphocyte proliferative responses of normal individuals and human filariasis patients (Wadee et al., 1987). Treatment with DEC or ivermectin clears MF and restores the immune responsiveness to filarial antigens in most individuals (Piessens et al., 1981; Lammie et al., 1992). However, ivermectin treatment in jirds does not restore proliferative cell responses or the PGRN (Brosshardt et al., 1995) indicating that adult worms may be implicated in filarial-induced hypo responsiveness. Maizels and Lawrence (1991) argue that both MF and adult parasites induce a state of immune tolerance that allows parasite survival and prevents progression to disease.

Cellular and humoral immune responses against different parasite stages have been studied primarily in the mouse model. Lawrence et al. (1994) observed contrasting cytokine and Ig isotype responses induced by MF versus long-lived adult worms inoculated intraperitoneally in BALB/c mice. MF infection resulted in strong INF-γ response that remained
elevated throughout the 28 days of the experiment. IgG1, IgG2a, IgG2b, and IgG3 levels were high throughout infection and there was no increase in IgE. Female worms induced stronger Th2 cell responses that male worms. IFN-γ and IL-2 were not detected, but IL-4 was present in high levels and was found to be produced by CD4+T cells. IgE and IgG1 were also elevated. These results indicate than adult worms, particularly females, exert a strong and rapid polarization of the immune response towards a Th2 phenotype, modulating the Th1 response induced by MF alone (Lawrence et al., 1994).

Similar results have been observed by Pearlman et al. (1993a) during early time periods of intraperitoneal MF infection. However, these authors found that prolonged exposure to MF also results in a Th2 response.

It is known that under certain not well understood circumstances, such as TPE, MF appear to act as a potent inflammatory stimulus. However, pathological damage of lymphatic vessels has been primarily attributed to the presence of adult worms (Von Lichtenberg, 1987; Jungmann et al., 1992), rather than to MF. Nevertheless, histopathological studies in the jird (Vincent et al., 1980; Klei et al., 1982; Jeffers et al., 1987), ferret (Crandall et al., 1982) and observations in humans (Meyers et al., 1976) have found MF within lymphatic granulomas, indicating that this stage may initiate or enhance inflammatory reactions in lymphatics and surrounding tissues.
MF have been shown to release prostaglandins E<sub>2</sub> (Liu et al., 1992) and other studies have established that MF of *B. malayi* incorporate exogenous arachidonic acid (Longworth et al., 1985). PGE<sub>2</sub> and prostacyclins are vasodilators, highly active inhibitors of platelet aggregation and endothelial adhesion, and potent immune modulators of leukocyte responses (Taffet and Russell, 1980; Snyder et al., 1982; Ellner and Spagnuold, 1986). Secretion of prostanoid products by MF may help the parasite to circulate intact throughout the bloodstream (Liu et al., 1992). PGE<sub>2</sub> has been reported to selectively inhibit IgE and enhance IgG4 synthesis from human B lymphocytes (Kimata et al., 1991). IgG4 may be very important in parasitic infections preventing immunopathology by several suppressive mechanisms (Hussain et al., 1987). It has been postulated that DEC works by altering arachidonic acid metabolism in MF and in host cells resulting in entrapment of the parasite in the microvasculature and initiation of inflammatory reactions (Liu and Weller, 1990; Kanesa-Thasan et al., 1991; Maizels and Denham, 1992).

In a study using immunoblotting of sera from endemic normals and hyporesponsive microfilaremic individuals, it was found that a 43 kD antigen derived from infective larvae was preferentially recognized by endemic normal individuals (Freedman et al., 1989). Cloning and characterization of this molecule has revealed that it is present primarily in L3 and MF, but not in adult worms, suggesting that larval stages
may be important in induction of protective immunity (Raghavan et al., 1994).

OBJECTIVES

Progress in understanding the immune regulation in lymphatic filariasis is dependent on laboratory animal models. The jird possesses distinct advantages for this purpose. Parasitological, pathological and immunological events during *B. pahangi* infection have been well defined in the jird and many aspects of the infection in this model mimic the human disease. The relatively inexpensive availability of the jird as inbred rodent along with the recent development of immunological reagents and assays enhances the usefulness of this animal model for the study of filariasis.

The overall objective of the research described in this dissertation is to further define the role of specific parasite stages in the granulomatous inflammatory response induced by *B. pahangi*.

The specific objectives are as follow:

Objective 1

To determine and compare the kinetics of the systemic and peritoneal inflammatory responses during intraperitoneal infection of *B. pahangi* evaluating the parasite stage specificity of these responses.
Objective 2

To determine the effect of low and high parasite burdens of different parasite stages in the peritoneal cavity on the systemic and peritoneal inflammatory response.

Objective 3

To compare the peritoneal and systemic inflammatory responses induced by parasites irradiated with increasing doses of gamma radiation, evaluating the effect of parasite development on the induction of inflammatory responses.

Objective 4

To determine the state of macrophage activation during the course of intraperitoneal infections with different parasite stages.
CHAPTER 2. DIFFERENTIAL INDUCTION AND REGULATION OF INFLAMMATORY RESPONSES BY LIFE CYCLE STAGES OF BRUGIA PAHANGI.

INTRODUCTION

Infection with the human lymphatic dwelling filarial parasites *Wucheraria bancrofti*, *Brugia malayi*, and *Brugia timori* leads to a complex spectrum of clinical outcomes that have been related to polarized filarial-specific immune responses (Ottesen, 1980). These immune responses range from a complete hyporesponsiveness to parasite antigen which is seen in most asymptomatic microfilaremic individuals to the hyperresponsiveness observed in individuals with lymphatic pathology and tropical pulmonary eosinophilia. Individuals who are equally exposed to infected mosquitoes but show no signs of infection, so called "endemic normals", are presumed to have developed a protective immunity (Ottesen, 1980; Ottesen, 1992; Nutman, 1995).

The parasite and host factors that determine the manifestations and progression of the different clinical outcomes are not well understood. However, it has been hypothesized that the various developmental stages of the parasite induce different responses and are in part responsible for these diverse clinical manifestations (Maizels and Lawrence, 1991). Microfilariae (MF) have been implicated as an important determinant in the state of immune hyporesponsiveness observed in most infected individuals who are asymptomatic. The vast majority of microfilaremic
asymptomatic patients exhibit suppressed B and T cell responses to filarial antigen (Nutman et al., 1987b; King et al., 1992; King et al., 1993). The microfilaricidal drugs diethylcarbamazine (Piessens et al., 1981; Lammie et al., 1988) and ivermectin (Lammie et al., 1992) restore responsiveness in the majority of the microfilaremic asymptomatic individuals. Conversely, inflammatory changes in the lymphatic vessels are mostly attributed to local immune responses directed towards adult worms (Von Lichtenberg, 1987; Jungmann et al., 1992; Nutman, 1995) and protective immunity has been hypothesized to be targeted to early larval stages, primarily L3 (Day, 1991).

The jird (Mongolian unguiculatus) infected with B. pahangi is an useful model to study filaria-mediated downregulation of the immune response. Jirds develop persistent microfilaremia associated with a state of immune hyporesponsiveness similar to that observed in microfilaremic asymptomatic human patients. In the jird, the loss of filarial specific responsiveness is characterized by decreased antigen-induced proliferative responses of spleen cells (Lammie and Stephen, 1983; Klei et al., 1990) and renal lymph node cells (Rao et al., 1995), and downregulation of granulomatous responses around antigen-coated beads embolized in the lungs (PGRN) (Klei et al., 1981; Klei et al., 1990). However, maintenance of the hyporesponsive state is not dependent on MF, since jirds with occult infections still
show downregulation and treatment of microfilaremic jirds with ivermectin removes MF but does not restore immune responsiveness (Brosshardt et al., 1995; Lin et al., 1995).

In the jird model, the pathologic and immunologic events that occur in the host after a primary subcutaneous infection have been defined. Although the chronology of the filarial life cycle in the jird is well known, the contribution of each specific filarial developmental stage to the host responses is still not clear. The objective of the present chapter is to better discern the influences that different life cycle stages of *B. pahangi* may have in the host inflammatory responses. *Brugia spp.* have been shown to develop normally and induce systemic immune responses when implanted into the peritoneal cavity (McCall et al., 1973; Klei et al., 1981; Jeffers et al., 1987; Klei et al., 1987). Further, all stages found in the vertebrate host can be recovered from this site and transplanted into other animals. This transplantation system was used to study worm-cell interactions and kinetics of parasite stage-induced inflammation.

**MATERIAL AND METHODS**

**Animals**

Each treatment group consisted of inbred, 6 to 8 week old, male, Mongolian jirds (*Meriones unguiculatus*). Animals were obtained from Tumblebrook Farms (West Brookfield, MA) and were maintained on standard rodent chow and water *ad libitum*. 
Parasites

*B. pahangi* L3 were harvested by crushing cold-anesthetized *Aedes aegypti* followed by sedimentation in a Baermann apparatus as previously described (Klei et al., 1990). 4th stage larvae were obtained from the peritoneal cavities of jirds that had been infected with L3 for a period of 14 days. Mature adult male and female worms were harvested from jirds with patent (> 60 DPI) intraperitoneal (IP) infections. Parasites were collected by repeated peritoneal lavages with phosphate buffered saline (PBS) pH 7.4. Worms were sexed, washed in HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffered RPMI-1640 medium (GIBCO, Grand Island, NY) containing penicillin (100 units/ml) and streptomycin (100 ug/ml) and transferred into syringes containing 2 to 3 ml of fresh medium. Single sex inoculations were performed using 16 gauge needles. MF obtained from peritoneal washes of jirds with chronic patent infections were separated by Percoll density centrifugation (Chandrashekar et al., 1984) and filtration through a 25 mm diameter 8 μm Nucleopore membrane (Millipore Corporation, Bedford, MA 01730) (Ponnudurai et al., 1975). MF could not be separated from eggs by this method. Therefore, both MF and eggs were used in the infections. Dead adult parasites were used in another experiment. Mature worms recovered from the peritoneal cavity were killed by 5 successive cycles of rapid freezing and thawing.
Parasite antigen:

Adult worms were collected from the peritoneal cavity of infected patent jirds and antigen extracts were prepared as previously described (Klei et al., 1988). Briefly, frozen, mature adult female and male *B. pahangi* were cut and disrupted in a Tenbrooke tissue grinder in PBS containing 2.5% Aprotinin (Sigma Chemical Co., St. Louis, MO). Parasite soluble extracts (SAWA) were filter sterilized and stored at -70°C.

Lung granulomas

A pulmonary granulomatous response (PGRN) was induced by the embolization of Cyanogen bromide Sepharose 4B (CNBr-4B) beads (Sigma Chemical Co, St. Louis, MO) in the lungs as previously described (Klei et al., 1988). Briefly, sized CNBr-4B beads were coated with SAWA or with diethanolamine (DEA) (Sigma), and were inoculated into the retro-orbital sinus 3 days prior to necropsy. Each jird received 2.5 x 10⁴ beads diluted in 0.5 ml of 0.05% saline. At necropsy, lungs were perfused with 10% formalin via the trachea. Tissues were embedded in paraffin and step sectioned at 2.5 µm. Several sections from each lung were cut at 50 µm intervals and were stained with hematoxylin and eosin. Areas of 20 granulomas around 40 to 60 µm diameter beads were measured in each animal using a image analysis system (Bioquant IV, Biometrics, Nashville, TN).
Samples collected:

Animals were bled from the retro-orbital plexus prior to euthanasia. MF counts were determined in 20 ul of fresh blood. The peritoneal cavity of each jird was washed extensively with PBS. Peritoneal washes were collected and cells were concentrated by centrifugation. Total peritoneal cell counts were determined in an automated counter, model ZF (Coulter Electronics, Inc, Hialeah, FL) after diluting peritoneal samples 1:500 in Isoton II coulter balanced electrolyte solution (Coulter Corporation, Miami, FL). Two cytospin preparations were made of each sample and stained with either a modified Giemsa stain or a non-specific esterase (NSE) stain (Yam et al., 1971). NSE stain was used to obtain a more accurate measurement of peritoneal macrophage numbers. MF were counted in 20 ul of individual peritoneal wash concentrated to 1 ml and are expressed as the mean MF per abdominal cavity. Peritoneal washes were searched for worms. Carcasses were soaked in PBS for at least 1 hour to recover remaining worms.

Statistical Analysis:

Significant differences between group means were tested with a comparative analysis of variance using Tukey's Studentized Range test. Results were considered significant when P values < 0.05 were obtained.
RESULTS

Kinetics of the PGRN in jirds inoculated intraperitoneally with L3.

All previous studies utilizing PGRN to measure systemic granulomatous inflammation to parasite antigen have been conducted in jirds infected via the subcutaneous route (Klei et al., 1990). Following this procedure parasites develop primarily in the lymphatics (Ash, 1973). The purpose of this experiment was to demonstrate that the kinetics of the PGRN in intraperitoneal (IP) infections with L3 is similar to that described following subcutaneous infections (SC). Two groups of 24 animals were used. One group was inoculated IP with 75 L3 and the other group received medium IP containing mosquito debris recovered from L3 harvest. Necropsies were performed at 7, 28, 56 and 181 days post-infection (DPI).

The granulomatous response induced in the lungs by SAWA-coated beads (PGRN) in the L3 infected group peaked at 28 DPI ($P < 0.005$) and decreased progressively after that point (Fig.2.1.). At 181 DPI PGRN of L3 infected animals was not significantly different from the PGRN of uninfected animals that received antigen-coated beads, or from infected or uninfected animals that received DEA-coated beads. Control animals receiving DEA- or antigen-coated beads had, at any time period, granuloma areas less than 7,000 $\mu$m$^2$.

The macrophage (MAC) was the cell that accumulated in higher numbers in the peritoneal cavity of L3 infected
Figure 2.1. Pulmonary granulomatous response to *Brugia pahangi* antigen-coated beads in jirds inoculated IP with L3. PGRN of infected and uninfected animals to DEA-coated beads is not shown, but was not significantly different from PGRN of uninfected jirds to *B. pahangi* antigen represented as control in this figure or from PGRN of L3 infected jirds to antigen at 181 DPI (P < 0.05). PGRN is measured as \( \mu m^2 \). Bars represent ± 1 standard deviation of the mean.
animals and was used as primary measurement of the peritoneal inflammatory response. Other cell types observed in the cytospins of peritoneal lavages from infected animals included eosinophils, lymphocytes, mast cells and neutrophils. In the L3 infected animals, peritoneal MACs were elevated throughout the infection period and peaked at 56 and 181 DPI (P < 0.05) (Fig. 2.2.). Peritoneal eosinophils increased markedly after 7 DPI and remained elevated throughout the infection (P < 0.05). Peritoneal eosinophils in control animals were absent or present in minimal numbers (Fig. 2.3.). Peritoneal lymphocytes in the L3 infection increased at 28 and 56 DPI (P < 0.05) and decreased to levels of controls at 181 DPI. Peritoneal mast cells from infected animals did not vary significantly from controls. Peritoneal neutrophils increased in infected animals at the acute time periods. A few, small (about 1 mm in diameter), peritoneal granulomas were found covering the serosal surface of liver and stomach in some of the L3 infected jirds at 181 DPI.

Parasite recovery decreased throughout infection until 56 DPI. In the L3 infected group, MF were present in large numbers in the abdominal lavages at 56 and 181 DPI (Table 2.1). 4 out of 6 animals infected with normal L3 had MF in the peripheral blood at 181 DPI.
Figure 2.2. Peritoneal macrophages in jirds inoculated IP with L3 *B. pahangi*. Macrophages are expressed as mean number of non-specific esterase (NSE) positive cells per peritoneal cavity. Note the increase of NSE + cells as infection with L3 progressed. Greatest number of macrophages accumulated at 56 and 181 DPI in the L3 infection. Low numbers of macrophages were recovered from uninfected animals throughout the infection. Bars represent the standard deviation of the mean.
Figure 2.3. Peritoneal eosinophils in jirds inoculated IP with L3 *B. pahangi*. Eosinophils are expressed as mean number per peritoneal cavity. Number of eosinophils in L3 infected animals are markedly increased after 7 DPI when compared with controls. Bars represent standard deviation of the mean.
Table 2.1. Worm Recovery and microfilaria counts in the peritoneal cavity of jirds inoculated IP with L3.

<table>
<thead>
<tr>
<th>DAYS POST INFECTION</th>
<th>WORM RECOVERY</th>
<th>PERITONEAL MICROFILARIAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 DPI</td>
<td>16.5 ± 7.97</td>
<td>-</td>
</tr>
<tr>
<td>28 DPI</td>
<td>15.5 ± 10.56</td>
<td>-</td>
</tr>
<tr>
<td>56 DPI</td>
<td>4.5 ± 3.33</td>
<td>29175 ± 21936</td>
</tr>
<tr>
<td>181 DPI</td>
<td>9.7 ± 5.71</td>
<td>354528 ± 210411</td>
</tr>
</tbody>
</table>

*Results are expressed as mean ± standard deviation.*
Comparison of the peritoneal and systemic inflammatory response in jirds inoculated with L4, male or female adult worms.

The hypothesis that downregulation of the PGRN is not dependent on MF was tested by comparing the PGRN response to antigen-coated beads in jirds receiving infections of female worms which produced MF to that of male worms. PGRN to parasite antigen was also investigated in jirds inoculated with L4; in order to compare the effect of this pre-adult stage on the kinetics of the inflammatory response with that induced by infections of adult parasites. Peritoneal inflammatory response induced by the different parasite stages was also evaluated. Four groups of 40 jirds each was inoculated IP with 26 L4, 12 adult male, 12 adult female worms or medium, respectively. The PGRN and peritoneal inflammatory response to these infections were compared. Necropsies were performed at 7, 14, 28, 56 and 166 DPI.

The PGRN in the female and male worm infected animals was elevated at 7 and 14 DPI. In the L4 infected group granuloma areas at 7 DPI were significantly smaller (P < 0.005) than those induced by the female or male infection at the same time period (Fig. 2.4.), but increased markedly at 14 DPI. After 14 DPI there was a trend of PGRN decrease in all infected groups. At 56 and 166 DPI the PGRN of all infected groups was markedly downregulated and not significantly different (P < 0.05) from the PGRN of infected
Figure 2.4. Pulmonary granulomatous response to parasite antigen in jirds inoculated IP with male, female or L4 *B. pahangi*. Granuloma areas are expressed in μm². Note the downregulation of the granulomatous response in all the infected groups at the chronic time periods (56 to 166 DPI). Bars represent one standard deviation of the mean.
animals to DEA or from the PGRN of uninfected animals to DEA and antigen. Furthermore, PGRN at 56 was not significantly different from the PGRN at 166 DPI in any group.

MACs accumulated in large numbers in the abdominal cavities of all infected jirds. However, differences were seen in the kinetics and numbers of cells present. In the female worm infected group MAC numbers increased above controls after 7 DPI and peaked at 28 DPI remaining elevated throughout the experiment (P < 0.001) (Fig. 2.5.). In the L4 infected group peritoneal MAC numbers increased above controls after 7 DPI and continued to increase to become highest at 166 DPI (P < 0.05). In the male worm infected group, MACs were elevated at 28 and 56 DPI (P < 0.005), and in the remaining time periods their numbers were not different from uninfected controls. Peritoneal eosinophils peaked at 56 DPI in the adult female worm (P < 0.001) and L4 (P < 0.05) infected groups (Fig. 2.6.). In the female worm infection peritoneal eosinophils decreased significantly (P < 0.001) at 166 DPI. Peritoneal eosinophils in the male worm infection increased above controls only at 28 DPI (P < 0.05). Granuloma formation on peritoneal surfaces was not observed grossly in any of the animals.

Female worm recovery decreased after 7 and 14 DPI (P < 0.05) and did not differ significantly thereafter. L4 and male worm recovery did not differ significantly throughout infection (Table 2.2.).
Figure 2.5. Peritoneal macrophages in jirds inoculated with male, female or L4 *B. pahangi*. Greatest macrophage accumulation is present in animals inoculated with either L4 or female worms. Bars represent standard deviation of the mean.
Figure 2.6. Peritoneal eosinophils in jirds inoculated with male, female or L4 *B. pahangi*. Greatest eosinophil accumulation is induced by L4 and female worms. Bars indicate standard deviation of the mean.
Table 2.2. Parasite recovery in jirds inoculated with male, female or L4 *B. pahangi*.

<table>
<thead>
<tr>
<th>DAYS POSTINFECTION</th>
<th>MALE WORM INFECTION</th>
<th>FEMALE WORM INFECTION</th>
<th>L4</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 DPI</td>
<td>5.88 ± 3.95</td>
<td>9 ± 1.22</td>
<td>8.25 ± 5.7</td>
</tr>
<tr>
<td>14 DPI</td>
<td>3.13 ± 2.66</td>
<td>6.13 ± 2.5</td>
<td>4.4 ± 2.12</td>
</tr>
<tr>
<td>28 DPI</td>
<td>4.63 ± 3.57</td>
<td>5.6 ± 2.8</td>
<td>1.86 ± 1.8</td>
</tr>
<tr>
<td>56 DPI</td>
<td>2.5 ± 2.29</td>
<td>3.9 ± 2</td>
<td>3.6 ± 4.9</td>
</tr>
<tr>
<td>166 DPI</td>
<td>3.14 ± 2.6</td>
<td>1.12 ± 0.8</td>
<td>5 ± 2</td>
</tr>
</tbody>
</table>

* Results are expressed as mean ± standard deviation.
Microfilariae were present in the abdominal cavity at all time periods in the adult female infected group, peaking at 56 DPI ($P < 0.005$). In the L4 infection, peritoneal MF were found only at 56 and 166 DPI (Fig. 2.7). No MF were present in peripheral blood in any of the infected groups at any of the necropsy time periods.

**Effect of dead worms on the granulomatous response.**

The hypothesis that living worms are needed to downregulate the granulomatous response was tested by comparing the PGRN of jirds inoculated with dead worms to that of jirds inoculated with the same number of viable parasites. Two groups of 15 animals were inoculated IP with 5 dead female worms and 5 dead male worms or with the same number and sex of living worms. A control group was inoculated with medium. Necropsies were performed on 5 animals per group at 15, 57 and 108 DPI.

Animals inoculated with dead worms had elevated PGRN to SAWA-coated beads at all time periods. Animals that were infected with living worms had at 15 DPI large granuloma areas not significantly different from dead worm inoculated animals. Granuloma areas decreased progressively in the living worm inoculated animals to become similar to control animals at 108 DPI (Fig. 2.8.).
Figure 2.7. Peritoneal microfilaria counts in jirds inoculated with female worms or L4. MF are expressed as total number per peritoneal cavity.
Figure 2.8. Pulmonary granulomatous response to parasite antigen in jirds inoculated with dead or living worms. Downregulation of the granulomatous response was induced by living but not dead worms. Granuloma areas are expressed in μm². Bars represent standard deviation of the mean.
Effect of parasite number on the peritoneal and systemic inflammatory responses.

The effect of different parasite burdens on the peritoneal and systemic inflammatory responses was tested. Jirds in four groups of 20 animals each were infected IP with 30 adult male worms, 5 adult male worms, 30 adult female worms or 5 adult female worms. Jirds in a control group were inoculated with medium. Necropsies were performed at 7, 14, 56 and 167 DPI. 15 animals in another group were each inoculated IP with a mixture of 10,000 MF and 27,000 eggs. Necropsies for this latter group were performed at 7, 14 and 56 DPI.

Pulmonary granuloma areas induced by SAWA-coated beads in animals inoculated with MF, 30 adult male, 30 adult female, 5 adult male, or 5 adult female worms were greatest at 7 and 14 DPI (P < 0.05) (Figs 2.9 and 2.10). At these early time periods the PGRN of groups inoculated with 30 female worms was less than that of the group inoculated with 5 female worms (P < 0.007) (Fig. 2.9). At chronic time periods, however, the parasite burden did not effect the level of PGRN downregulation. The PGRN induced by 5 or 30 female worms at 56 DPI was not significantly different from controls. However, PGRN induced by MF, 5 male or 30 male worms at 56 DPI was greater than the PGRN of controls (P < 0.01). Different doses of male worms did not effect the PGRN (Fig. 2.10).

MAC numbers in the peritoneal cavity increased more pronouncedly throughout the infection in the animals that
Figure 2.9. Comparison of the pulmonary granulomatous response induced in jirds with IP inoculation of high and low numbers of female worms as well as MF. 30 female worms induced significantly smaller granuloma areas than 5 female worms at 7 and 14 DPI (P < 0.05). Downmodulation of the PGRN occurred at 56 and 167 DPI in animals inoculated with different numbers of female worms or at 56 DPI in animals inoculated with MF. Granuloma areas are measured in μm². Bars represent standard deviation of the mean.
Figure 2.10. Comparison of the pulmonary granulomatous response in jirds inoculated IP with high and low male worm numbers. No significant difference in PGRN was found in jirds inoculated with 30 or 5 numbers of male worms. Bars represent standard deviation of the mean.
received 30 adult female worms (data not shown). A few (2 or 3) macroscopic granulomas (about 1 mm in diameter) were found on the serosal surface of liver and stomach, as well as in the omentum of some of the animals inoculated with 30 female worms at 56 and 167 DPI.

The parasite recovery of animals inoculated with 30 female adult worms was significantly greater than the recovery of animals inoculated with 5 female worms only at 7 DPI (P < 0.05) (Table 2.3.). The parasite recovery of animals inoculated with 30 male worms was significantly higher than in animals inoculated with 5 male worms at all time periods (P < 0.05).

Peritoneal MF counts in the 30 female worm infection were significantly increased over those in the 5 female worm infection at 56 and 167 DPI (P < 0.05), but not at 7 or 14 DPI. The MF recovery in jirds inoculated with MF was very low and significantly decreased over time when compared with the MF recovery in the female worm infections (Fig. 2.11.).

**DISCUSSION**

Induction and maintenance of the hyporesponsive state in the jird is not uniquely dependent on the presence of MF. This is consistent with previous indirect observations (Brosshardt et al., 1995; Lin et al., 1995). In the present study all life cycle stages induced an early systemic granulomatous response to parasite antigens as measured by the artificial lung granuloma model. PGRN response was downmodulated as infection progressed to the chronic time
Table 2.3. Parasite recovery from jirds inoculated with different numbers of adult worms*.

<table>
<thead>
<tr>
<th>DAYS POSTINFECTION</th>
<th>30 MALE WORMS</th>
<th>5 MALE WORMS</th>
<th>30 FEMALE WORMS</th>
<th>5 FEMALE WORMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 DPI</td>
<td>20.4 ± 2.7</td>
<td>3 ± 1.67</td>
<td>23.7 ± 4.4</td>
<td>2.6 ± 1.5</td>
</tr>
<tr>
<td>14 DPI</td>
<td>17.6 ± 7</td>
<td>4.4 ± 0.8</td>
<td>5.5 ± 2.5</td>
<td>1.6 ± 1.6</td>
</tr>
<tr>
<td>56 DPI</td>
<td>17 ± 6.27</td>
<td>3 ± 1.26</td>
<td>4.25 ± 3.9</td>
<td>0.6 ± 1.2</td>
</tr>
<tr>
<td>167 DPI</td>
<td>9.8 ± 8.3</td>
<td>3.2 ± 1.16</td>
<td>0.5 ± 0.5</td>
<td>0</td>
</tr>
</tbody>
</table>

* Results are expressed as mean ± standard deviation.
Figure 2.11. Peritoneal microfilaria counts in jirds inoculated with different female worm numbers or with MF. Note the elevated numbers of MF present in jirds with inoculations of 30 female worms.
period in the groups that were inoculated with male, female, L4, L3 or MF. This suggests that parasite factors responsible for the induction of the downregulation are present in multiple parasite stages.

Inoculation of dead adult worms did not induce granulomatous downregulation and pulmonary granulomas remained large at all time points following dead worm inoculation. This indicates that living worms are needed to induce a filarial-specific hyporesponsive state and that parasite molecules relevant to immunomodulation may be products of worm metabolism. A number of these immunomodulatory molecules have been described (Wadee et al., 1987; Lal et al., 1990). Alternatively, it is possible that inflammatory downmodulation requires constant and cumulative release of parasite antigens which would only occur if the worms are alive. This would suggest that antigenic load produced by chronicity is important in the state of immune hyporesponsiveness.

Our results show that the degree of PGRN was modified by different parasite burdens. Significant difference in the PGRN between inoculation of different female worm numbers was found early in infection, but not at the chronic time periods. No significant differences were found in groups infected with different numbers of adult male worms, which would suggest the existence of separate factors in male and female worms which effect immunoregulation. Alternatively,
it is possible that the female worms produce more of the same immunoregulatory factors. Interestingly, inoculation of MF alone induced downregulation of the PGRN at 56 DPI. Moreover, this downmodulation was less pronounced than in the female and male worm infected groups at the same time period. The lighter antigenic load of MF inoculated in a single bolus could again explain this difference. The effect of different MF burdens alone, however, was not tested. Other studies in the *B. pahangi*-infected jird have found significant correlation between the degree of PGRN downregulation and the levels of circulating MF and female worm recovery suggesting that the parasite burden positively influences the degree of hyporesponsiveness (Brosshardt et al., 1995). Petit et al. (1993) found a correlation between the PGRN and levels of circulating MF in jirds with similar adult worm burdens. Immunological observations in human patients infected with filariae have consistently found diminished parasite-specific lymphocyte proliferative responses in individuals with circulating MF when compared to microfilaremic subjects (Ottesen et al., 1977; Piessens et al., 1980; Nutman et al., 1987a; King et al., 1992). Studies of cytokine profiles have shown that the asymptomatic microfilaremic group of patients have a shift toward a predominantly Th2 response that contrast with the Th1-like response of chronic pathology patients (King et al., 1993; Maizels et al., 1995). Detection of cytokine mRNA in spleen and lymph nodes from
B. pahangi-infected jirds have demonstrated a pattern similar to that found in human patients. mRNA for IL-10 and IL-4 are found in the jird coinciding with the state of hyporesponsiveness that occurs as the infection progresses to the chronic period (Mai, 1996). These Th2 cytokines are known to possess immunomodulatory and antiinflammatory properties (Mosmann and Moore, 1991) that would prevent development of pathology and favor persistence of the parasites. Experiments in the mouse model have demonstrated that IP inoculated adult worms rapidly stimulate a Th2 cell response, whereas MF induces a immediate Th1 cell response (Lawrence et al., 1994). However, prolonged IP exposure to MF and repeated (but not single) SC inoculations of MF antigen also induce a Th2 phenotype (Pearlman et al., 1993a; Pearlman et al., 1993b). The mechanisms that drive the expansion of a Th2 response are not well understood, but chronicity and high load of antigen have been shown to be important (Bretscher et al., 1992; Parish and Liew, 1972). Other factors that have been implicated in induction of different Th subsets include APC (Gajewski et al., 1991), costimulatory molecules (Weaver et al., 1988), Th subset-specific antigen (Scott et al., 1988; Liew et al., 1990) and the initial cytokine milieu (Seder et al., 1992). Filarial antigens may exist that activate preferentially specific Th2 subsets. It is also possible that certain filarial molecules trigger an initial release of Th2 cytokines by non T cell,
creating the cytokine milieu appropriate for Th2 proliferation and downregulation of proinflammatory cytokines. Our data suggest that if such immunomodulatory molecules exist they are shared by various parasite stages.

Interestingly, peritoneal inflammatory response, primarily characterized by MAC accumulation, did not correspond to the pulmonary granulomatous response. The largest numbers of peritoneal MACs were present at the chronic time period in infections with L4, adult female worms or L3. This elevation in MAC numbers was associated with the presence of large numbers of peritoneal MF and low pulmonary granulomatous responses. Conversely, infections that did not result in production of numerous viable MF, such as inoculations of adult male worms, MF or adult dead worms, induced low levels of peritoneal MACs. These data suggest that MF may be a potent inflammatory stimulus when compartmentalization phenomena occur, and result in localized reactions independent of the systemic response.

The magnitude of the intraperitoneal inflammatory reaction and the feasibility of inoculating different life cycle stages into the peritoneal cavity makes this site an ideal body compartment to study cell-parasite interactions during \textit{B. pahangi} infection (Klei et al., 1981; McCall et al., 1973; Jeffers et al., 1987). Intraperitoneal inoculations are comparable to subcutaneous infections in that the cellular composition of the inflammatory response caused by
B. pahangi is similar in both peritoneal cavity and lymphatic vessels (Jeffers et al., 1984; Jeffers et al., 1987). Previous studies have also shown that preexistent intraperitoneal infections result in decreased granulomatous lymphatic lesions after subcutaneous inoculation (Klei et al., 1987). Present results show that the PGRN to worm antigen follows similar kinetics following L3 inoculation in both body locations. Inoculation of L3 IP can downregulate the systemic inflammatory response in the same manner as L3 that localize in the lymphatic system (Klei et al., 1981; Rao et al., 1995). This suggests that the IP implantation of adult stages can also mimic specific responses that occur in the natural location of filariae.

In conclusion, viable worms are necessary to induce and maintain the hyporesponsive state, but MF alone are not responsible for this condition. Our experiments also suggest that parasite burden is important in downmodulation of the inflammatory response. Further investigations are necessary to determine whether early larval stages, the L3 and L4 stages, are involved in the downregulation of the granulomatous response in the jird. Attempts were made to address this question in chapter 3.
CHAPTER 3. EFFECT OF GAMMA RADIATION ON BRUGIA L3 DEVELOPMENT IN VIVO AND THE KINETICS OF GRANULOMATOUS INFLAMMATION INDUCED BY THESE PARASITES

INTRODUCTION

Lymphatic filariasis is a parasitic disease caused by Wuchereria bancrofti, Brugia malayi and B. timori that affects 120 million people in tropical and subtropical regions (WHO, 1992; Ottesen and Ramachandran, 1995). The disease has a complex spectrum of clinical outcomes that have been related to different immune responses of the host to filarial antigen (Ottesen, 1980; Piessens et al., 1980; Nutman et al., 1987a; Ottesen and Ramachandran, 1995). In endemic areas, the majority of the infected population is generally asymptomatic and microfilaremic. Pathology occurs in a smaller proportion of infected people, and interestingly most of these patients are amicrofilaremic. However, chronic lymphatic disease often results in dramatic disfiguring lesions. A smaller and not well studied group of individuals in the filarial spectrum are those that are thought to be putatively immune; so called endemic normals. The relatively polarized immune responses of microfilaremic asymptomatic individuals versus amicrofilaremic symptomatic patients have lead to the suggestion that the microfilaria stage is responsible for the immune hyporesponsiveness characteristic of most microfilaremic individuals. Studies in human and experimental animals have partly supported this
hypothesis (Piessens et al., 1981; Wadde et al., 1987; Lammie et al., 1988; Lammie et al., 1992). However, it is now clear that MF is not the only life cycle stage involved in the downregulation of the granulomatous response (Brosshardt et al., 1995; Lin et al., 1995), (Chapter 2). Scant work, however, has been done on the role that other life cycle stages play in the diverse immune responses to filaria.

The jird (*Meriones unguiculatus*) has been used extensively for the study of filariasis. Jirds chronically infected with *B. pahangi* develop an immune hyporesponsive state (Klei et al., 1981; Lammie and Stephen, 1983; Klei et al., 1990) that may be similar to that observed in human filariasis (Ottesen et al., 1977; Piessens et al., 1980; Nutman et al., 1987a; Maizels and Lawrence, 1991; King et al., 1992). This model is particularly advantageous to investigate the factors and mechanisms implicated in the induction and maintenance of this hyporesponsive state. The kinetics of the parasitology, pathology and immunologic events have been well characterized during primary subcutaneous infections with *B. pahangi* in male jirds. The timing of these events can be correlated to the periods of parasite development. An *in vivo* pulmonary granuloma model has been used to measure inflammatory responses induced by parasite antigen (Klei et al., 1981). Pulmonary granulomatous responses (PGRN) to soluble adult worm antigen (SAWA) peak at 14 days postinfection (DPI) and begin to
decrease before patency (56 DPI), when adult worms are still immature (Rao et al., 1995). Proliferative responses to SAWA of renal lymph node cells draining the site of infection follow kinetics that are similar to the PGRN (Rao et al., 1995). Spleen cell blastogenesis increases later and subsequently decreases. Lymphatic granulomas peak between 60 and 90 DPI and decrease significantly thereafter (Klei et al., 1988; Klei et al., 1990). Depressed immune and inflammatory responses in *B. pahangi*-infected jirds are maintained throughout the chronic period in association with a persistent microfilaremia. However, a small percentage of chronically infected jirds are amicrofilaremic. These, however, are not resistant to reinfection and do not respond to antigen indicating that the hyporesponsive state is maintained in the absence of MF (Brosshardt et al., 1995; Lin et al., 1995). Furthermore, chemotherapeutic removal of MF with ivermectin in jirds does not restore immune responsiveness (Brosshardt et al., 1995).

Intraperitoneal (IP) infections of *B. pahangi* in the jird mimic subcutaneous infections in that the cell components of the inflammatory response (Jeffers et al., 1987) and the kinetics of the PGRN are similar (Chapter 2). Parasite inoculation into the jird peritoneal cavity at any stage of development remain in this site, continue to develop normally and can easily be recovered (Malone et al., 1974). Thus, the peritoneal cavity has been used as an ideal
compartment to study the effect of different filarial life cycle stages on the peritoneal and systemic inflammatory responses. These experiments have demonstrated that viable worms are necessary to initiate and maintain the downregulation of the PGRN induced by SAWA-coated beads, and that this downregulated state is not dependent on MF (Chapter 2).

Gamma radiation is known to interrupt the normal development of nematodes and this effect is dependent on radiation dosage (Oothuman et al., 1978). To further clarify which life cycle stages induce filarial hyporesponsiveness, jirds were infected intraperitoneally with L3 irradiated with increasing doses of gamma radiation.

**MATERIALS AND METHODS**

**Animals and Experimental design:**

Inbred female jirds (*Meriones unguiculatus*) were obtained from Tumblebrook Farms (West Brookfield, MA). Animals were maintained on standard rodent chow and water *ad libitum*. Groups of 12 to 16 jirds were inoculated intraperitoneally with 100 L3 exposed or not to different doses of gamma radiation. An additional group of jirds was used as an uninfected control. Necropsies were performed on 3 or 4 jirds per group at 7, 14, 28 and 118 DPI.

**Parasites:**

L3 were collected from crushed *Aedes aegypti* mosquitoes after sedimentation in a Baermann apparatus as previously
described (Klei et al., 1990). Irradiation of L3 was carried out by exposure of 1 ml syringes containing 100 L3 in 0.5 ml HEPES buffered RPMI-1640 medium (GIBCO, Grand Island, NY) supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml) to a 60 Co source at 1447 krads/min. L3 were exposed to 5 different increasing levels of radiation. Radiation doses for each group were 0, 15, 25, 35, 45, and 90 krads, respectively.

Antigen:

SAWA used to coat beads was prepared from frozen, mature adult female and male B. pahangi worms by methods previously described (Klei et al., 1988). Adult worms were recovered from female jirds with patent IP B. pahangi infections (>60 DPI).

Measurement of lung granuloma areas:

Three days prior to necropsy jirds were inoculated in the retroorbital sinus with SAWA- or diethylamine-(DEA) coated CNBr-4B beads (Sigma Chemical Co, St. Louis, MO) that embolized in the lungs as previously described (Klei et al., 1988). Each jird received 2.5 x 10^4 beads diluted in 0.5 ml of 0.05% saline. At necropsy, lungs were perfused with 10% formalin via the trachea. Tissues were embedded in paraffin and step sectioned at 2.5 μ. Several sections from each lung were cut at 50 μm intervals and were stained with hematoxylin and eosin. Areas of 20 granulomas around 40 to 60 μm diameter beads were measured in each animal using a image
analysis system (Bioquant IV, Biometrics, Nashville, TN).

Samples collected:

Animals were bled prior to euthanasia from the retroorbital sinus. MF were counted in 20 µl of fresh blood. The peritoneal cavity of each jird was washed extensively with phosphate buffered saline (PBS). Peritoneal washes were collected and cells were concentrated by centrifugation. Total peritoneal cell numbers were determined with the aid of an automated counter (model ZF, Coulter electronics, Inc, Hialeah, Fla). Peritoneal samples were diluted 1:500 with Isoton II coulter balanced electrolyte solution (Coulter Corporation, Miami, FL). Peritoneal cell differentials were determined on cytospin preparations. Two cytospins were made of each sample and stained either with a modified Giemsa stain or with a non-specific esterase (NSE) stain (Yam, Li and Crosby, 1971). Peritoneal MF were counted of 20 µl of total concentrated peritoneal fluid and are expressed as the mean number collected per animal's peritoneal cavity. Worms were recovered from peritoneal washes. Carcasses were soaked for more than one hour and examined to recover remaining worms. Free floating peritoneal granulomas with or without trapped parasites were collected and fixed in 10% formalin fixative.

Microscopic pathology of peritoneal granulomas:

After fixation granulomas were dehydrated in a graded ethanol series to absolute ethanol and were then infiltrated
and embedded in Glycol Methacrylate (JB-4 Embedding Kit, Polysciences, Inc. Warrington, PA). Sections were cut at 2 to 3 μm with a glass knife and mounted on clean glass slide which were heated to 60°C to attach sections. Routine hematoxylin and eosin stain was performed on sections.

**Parasite measurements and development:**

Worm lengths were determined with the aid of a camera lucida by drawing the outline of the worms. Lengths of these drawings were measured with an image analysis system (Bioquant IV, Biometrics, Nashville, TN). Worm lengths were calculated in mm.

Parasite development and sex (when possible) were determined following morphological descriptions of *B. pahangi* stages previously reported (Schacher, 1962a).

**Statistical Analysis:**

Statistical comparisons were made between groups using the Tukey's Studentized Range Test for variability. Differences were considered significant with P values < 0.05.

**RESULTS**

**Parasite recovery.**

 Recoveries of parasites irradiated with 25, 35, 45 or 90 krad decreased significantly over time in comparison with normal parasites (P < 0.05) (Table 3.1.). The trend of decrease of irradiated parasites was inversely related to the doses of irradiation. Parasites irradiated with 45 or 90 krad were not recovered at 118 DPI.
Table 3.1. Parasite recovery from jirds inoculated IP with normal or irradiated L3 at different times post infection *.

<table>
<thead>
<tr>
<th>Radiation Doses</th>
<th>0 krads (4)</th>
<th>15 krads (3)</th>
<th>25 krads (3)</th>
<th>35 krads (3)</th>
<th>45 krads (3)</th>
<th>90 krads (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 DPI*</td>
<td>31 ± 21.6</td>
<td>45 ± 18.2</td>
<td>47 ± 18.6</td>
<td>13.3 ± 4.1</td>
<td>17.3 ± 3.9</td>
<td>17 ± 7.4</td>
</tr>
<tr>
<td>14 DPI</td>
<td>19 ± 10.7</td>
<td>17 ± 20.5</td>
<td>19 ± 11.34</td>
<td>6.3 ± 1.25</td>
<td>2.33 ± 1.7</td>
<td>1.7 ± 1.2</td>
</tr>
<tr>
<td>28 DPI</td>
<td>36 ± 17.7</td>
<td>25 ± 16.3</td>
<td>22.7 ± 21</td>
<td>7 ± 3.6</td>
<td>18 ± 18.8</td>
<td>4 ± 4.32</td>
</tr>
<tr>
<td>118 DPI</td>
<td>24 ± 16.7</td>
<td>5.7 ± 3.9</td>
<td>2 ± 1.4</td>
<td>0.33 ± 0.5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Results are expressed as mean number of parasite recovery ± standard deviation.

a Days post infection with *Brugia pahangi*.

() Number of animals in each necropsy time period within a treatment group.
Parasite measurements and development.

Worm lengths followed a trend similar to parasite recovery and were inversely related to radiation dose (Table 3.2.). Parasite lengths did not vary significantly among the different treatment groups at 7 DPI. However, lengths of irradiated parasites at 14, 28 and 118 DPI were significantly reduced when compared to those of non-irradiated parasites (P < 0.05). Gamma radiation at 35, 45 or 90 krads prevented larval molt to the adult stage. Some parasites irradiated with 15 or 25 krads developed into adult female worms and stunted larvae with features of L4. No MF were observed in the uteri of female worms, which developed following irradiation.

Pulmonary granulomatous response.

The PGRN peaked at 14 DPI in groups inoculated with normal L3 or L3 irradiated with 15, 35 and 45 krads (P < 0.05) (Fig. 3.1.). Downregulation of the PGRN was observed in groups that received non-irradiated parasites or parasites irradiated with 15 krads (P < 0.05). No significant decrease of PGRN occurred in groups that received parasites irradiated with more than 15 krads. Although a trend of decrease in PGRN after 14 DPI was observed in the animals that received parasites irradiated with 25 krads, this decrease was not statistically significant. Animals that received non-irradiated parasites had small granuloma areas at 118 DPI which did not differ from those of controls but were
Table 3.2. Lengths and development of *Brugia pahangi* exposed to increasing doses of irradiation or to no irradiation *.

<table>
<thead>
<tr>
<th>RADIATION DOSES:</th>
<th>0 krads</th>
<th>15 krads</th>
<th>25 krads</th>
<th>35 krads</th>
<th>45 krads</th>
<th>90 krads</th>
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<tbody>
<tr>
<td>7 DPI</td>
<td></td>
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<tr>
<td>1.9 ± 0.05</td>
<td>1.82 ± 0.02</td>
<td>1.81 ± 0.07</td>
<td>1.6 ± 0.05</td>
<td>1.62 ± 0.02</td>
<td>1.73 ± 0.04</td>
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<tr>
<td>L3 *</td>
<td>L3</td>
<td>L3</td>
<td>L3</td>
<td>L3</td>
<td>L3</td>
<td>L3</td>
</tr>
<tr>
<td>5.4 ± 0.36</td>
<td>4.5 ± 0.05</td>
<td>4.17 ± 0.2</td>
<td>3.5 ± 0.22</td>
<td>2.9 ± 0.3</td>
<td>2.1 ± 0</td>
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</tr>
<tr>
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<tr>
<td>10.3 ± 0.4 Male</td>
<td>7.6 ± 0.97</td>
<td>6.9 ± 0.12</td>
<td>4.8 ± 0.2</td>
<td>4.6 ± 1.9</td>
<td>2.85 ± 0.2</td>
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</tr>
<tr>
<td>12.5 ± 0.5 Female</td>
<td>L4, Female</td>
<td>Female, L4</td>
<td>L4</td>
<td>L4</td>
<td>L4</td>
<td>L4</td>
</tr>
<tr>
<td>118 DPI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15.7 ± 1.05 Male</td>
<td>23.3 ± 0.6</td>
<td>18.9 ± 1.25</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Female</td>
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<td>Female</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>38.8 ± 2.3 Female, MF</td>
<td></td>
<td></td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Results are expressed as mean of parasite lengths (mm) ± standard deviation.

a Developmental stages found at each time period in the different treatment groups are indicated.
Figure 3.1. Pulmonary granulomatous response to parasite antigen in jirds inoculated with normal L3 or with L3 exposed to increasing doses of gamma radiation. Mean values with different letters are significantly different (P < 0.05). Bars represent standard deviation of the mean.
significantly smaller than those of animals that received parasites irradiated with 15 krads (P < 0.05).

Peritoneal inflammatory response.

MAC accumulation was used as measurement of peritoneal inflammation. The MAC was the inflammatory cell type most abundant in peritoneal washes of infected and uninfected animals. Pronounced increase of peritoneal MACs was observed at 118 DPI in the group that received non-irradiated parasites when compared to any other group (P < 0.005) (Fig. 3.2.). However, peritoneal MAC numbers from animals that received irradiated parasites were not significantly different from uninfected controls. Other cells found in the peritoneal cytospins included eosinophils, lymphocytes, neutrophils and mast cells. Numbers of peritoneal eosinophils in animals receiving non-irradiated L3 were higher at 28 and 118 DPI when compared with the other infected or control groups (P < 0.05). Peritoneal eosinophils from jirds receiving irradiated L3 did not increase consistently throughout infection or among different treatment groups.

Granulomas partially or totally surrounding irradiated and non-irradiated parasites were found free floating in the peritoneal cavity (Fig. 3.3.). The majority of these granulomas were associated with dead or damaged worms. Histologically, these granulomatous lesions were characterized by collections of MACs and eosinophils, with
Figure 3.2. Peritoneal macrophages in jirds inoculated with normal L3 or L3 exposed to increasing doses of gamma radiation. Peritoneal macrophages are represented as non-specific esterase (NSE) positive cells. Bars represent standard deviation of the mean.
Figure 3.3  Photograph of a *B. pahangi* larva surrounded by a peritoneal granuloma after 14 days post infection.
fewer lymphocytes and neutrophils (Fig. 3.4.). These granulomas were sometimes accompanied by occasional foci of necrosis and/or organizing fibrous tissue deposition. Sections of dead or degenerating worms were found surrounded by this granulomatous infiltrate. Granulomas from jirds inoculated with normal L3 had MF at 118 DPI. At 7 and 14 DPI granulomas appear to occur more frequently in jirds inoculated with irradiated L3 than in those inoculated with non-irradiated L3. However, at 118 DPI granulomas were found only in jirds inoculated with normal L3. No differences in the cellular types or histopathological features of granulomas collected from different treatment groups could be discerned.

MF counts.

MF were present in large numbers (319.5 x 10³ ± 38.5 x 10³) at 118 DPI in all the animals that received non-irradiated parasites. One out of four animals that received non-irradiated L3 had MF in peripheral blood at 118 DPI. No MF were present in peripheral blood or peritoneal cavity of animals that received irradiated parasites.

DISCUSSION

Our experiments demonstrated that gamma radiation inhibits the growth and decreases the survival of B. pahangi as previously reported (Oothuman et al., 1978; Devaney et al., 1993). These authors also found no MF in female worms and few or no recognizable male worms. Similarly, parasite
Figure 3.4 Photomicrograph of the granulomatous inflammation induced by *B. pahangi* larva 14 days post infection. Macrophages and eosinophils are numerous and adhere to the surface of the degenerating L4.
survival and worm lengths appeared to be inversely related to the irradiation dose. Oothuman et al. (1978) described the effects of 10, 25 and 45 krads on L3 inoculated subcutaneously into cats. This report differs with ours in that 25 kads prevented the worms from developing beyond the early L4. In our experiment irradiation with 15 or 25 krads allowed development of some parasites into infertile female adults. These slight differences could be related to the different route of inoculation chosen, to differences in the responses that the jird and cat may have to *B. pahangi* or to variations in calibration of the radiation source.

Downregulation of the PGRN did not occur in animals that received L3 irradiated with 25, 35, 45 and 90 krads. However, non irradiated parasites or those irradiated with 15 krads induced downmodulation of the granulomatous response. These findings suggest that viable adult worms are important in the induction of a hyporesponsive state. Nevertheless, these observations do not rule out with certainty that the L4 stage may have some influence. Furthermore, the effect of parasite burden on the PGRN could not be discerned with clarity. Small numbers of viable female worms were found in animals that received L3 irradiated with 25 krads, but the PGRN was not significantly decreased. In addition, the degree of PGRN downmodulation was greater in the animals that received normal L3 than in animals that received L3 irradiated with 15 krads. This difference in the degree of
granulomatous downmodulation may be related to the presence of MF and/or the requirement of active adult worms not damaged by irradiation, or to higher total parasite burden. Results in Chapter 2 demonstrated that parasite burden effects the PGRN, since high numbers of female worms induced smaller granuloma areas than lower numbers. Studies by Brosshardt et al. (1995) showed that the degree of antigen-mediated granulomatous downregulation and the decrease in splenocyte proliferation correlated with the number of circulating MF and adult female worms indicating that parasite burden influences positively the degree of hyporesponsiveness.

It is very likely that MF contribute to filarial hyporesponsiveness as suggested by others (Klei et al., 1990; Brosshardt et al., 1995). Results in chapter 2 demonstrated that inoculation of MF alone induces downregulation of the PGRN. Prolonged IP exposure to MF or repeated (but not single) subcutaneous inoculations of MF antigen in mice result in a Th2 response (Pearlman et al., 1993a; Pearlman et al., 1993b), thereby indicating that chronicity and parasite burden may play a decisive role.

The lack of PGRN downmodulation in animals that received L3 exposed to high doses of irradiation may also be explained by the impaired release of certain antigens relevant in immunomodulation. It has been demonstrated that irradiation inhibits protein synthesis in *Schistosoma mansoni*
schistosomula (review in Wales and Kusel, 1992). Recent studies in *B. pahangi* did not reveal consistent differences in protein synthesis between irradiated and non-irradiated L3 or L4 (Devaney et al., 1993). However, the adult stage was not studied. The present results and data in chapter 2 support the importance of adult stages for inducing downmodulation in the absence of MF as other experiments corroborate (Brosshardt et al., 1995; Lin et al., 1995).

The profile of cytokine mRNA in jirds subcutaneously infected with *B. pahangi* follows a Th2 pattern that coincides with the hyporesponsiveness state characteristic of the chronic period in this model (Mai, 1996) and mimics the cytokine response reported in microfilaremic asymptomatic human patients (King et al., 1993; Maizels et al., 1995). It has been proposed that the downregulation of Th1 cytokines in filariasis diminishes cellular effectors that lead to pathology and clearance of parasites (Nutman, 1995). Vaccination studies in the jird with radiation attenuated-*B. pahangi* L3 showed that resistance is associated with increased severity of lymphatic lesions and higher PGRN (Petit et al., 1993). It is possible that the high PGRN associated with protection is mediated by a Th0 or Th1 phenotype.

Peritoneal inflammatory response, as measured by MAC numbers, was most pronounced at the chronic time period in jirds receiving the non-irradiated L3. This prominent
inflammatory response was associated with the presence of high numbers of intraperitoneal MF, indicating that MF may act in certain circumstances as a potent inflammatory stimulus. MF has been described in granulomatous lesions in the jird (Vincent et al., 1980; Klei et al., 1982; Jeffers et al., 1987), ferret (Crandall et al., 1982) and humans (Meyers et al., 1976). Free floating peritoneal granulomas in jirds inoculated with normal L3 were found more frequently at the time when MF were present. MF are most commonly found circulating in peripheral blood and throughout the microvasculature of diverse organs without inducing any apparent inflammatory response. Compartimentalization phenomena driven by localized immune responses yet to be defined may result in entrapment and killing of MF followed by granulomatous inflammation. In our study, the peritoneal inflammatory response did not correlate with the downregulated PGRN suggesting that factors inducing peritoneal inflammation exist independently of those responsible for systemic responses. Such difference in responses suggests that diverse body compartments differ in the ability to cope with the parasite, which may be related to the nature of parasite- and/or parasite product-cell interactions as well as particular cell types that are recruited to such body locations. This may also explain the differences in the kinetics of proliferative responses to SAWA of spleen, axillary lymph node and renal lymph node
cells from jirds inoculated subcutaneously and harboring adult parasites in lymphatic vessels that drain to renal lymph nodes (Klei et al., 1990; Rao et al., 1995). Variability in regional and systemic immune responses to parasite antigen have also been found in the dog infected with *B. pahangi* (Miller et al., 1991) as well as in infections with gastrointestinal nematodes (Kelly et al., 1991).

The peritoneal cavity appears to be an appropriate compartment to study parasite cell-interactions and may be very advantageous to characterize further immunological properties of cells such as MACs which participate in the immune and inflammatory response to filaria. The MAC is recruited in large numbers into the peritoneal cavity in response to *B. pahangi* infection and is also a central component of granulomatous lymphatic lesions and PGRN. However, scant information has been collected about the significance of the MAC in filarial infections. This will be further considered in chapter 4.
CHAPTER 4. MACROPHAGE ACTIVATION AND TNF PRODUCTION IN JIRDS INFECTED WITH FEMALE OR MALE BRUGIA PAHANGI

INTRODUCTION

_Wuchereria bancrofti_ and _Brugia malayi_ are lymphatic-dwelling filarial nematodes that infect humans in tropical and subtropical regions of the world. The pathology caused by filarial parasites is primarily characterized by a granulomatous inflammation to the parasite and parasite products that has been attributed to a state of specific filarial immune hyperresponsiveness (Lichtenberg, 1957; Ottesen, 1980). Lymphatic dilatation in absence of marked inflammation has been reported in microfilaremic asymptomatic patients (Jungmann et al., 1991) and has been reproduced in immunodeficient mice (nu/nu and SCID) (Vincent et al., 1984; Nelson et al., 1991). Lymphatic changes in absence of marked inflammation have been interpreted as an example of specific immunosuppression and are probably related to the persistent mechanical action of adult worms within lymphatic vessels (Von Lichtenberg, 1987). Immune reconstitution in nude mice with T cells from filarial sensitized immunocompetent mice results in development of a granulomatous reaction within and around lymphatics proving the importance of T lymphocytes in disease manifestations (Vickery et al., 1991). Intact worms are typically associated with lymphangiectasis, whereas damaged or dead worms are always accompanied by inflammation and lymphatic obstruction (Rifkin and Thompson, 1945; Wartman, 1947; Lichtenberg, 1957).
Immunological studies in microfilaremic asymptomatic human patients have revealed depressed lymphoproliferative responses (Ottesen et al., 1977; Piessens et al., 1980; Nutman et al., 1987a; King et al., 1992) to filarial antigen and production of Th2 cytokines (IL-4 and IL-10) (King et al., 1993; Maizels et al., 1995). Conversely, subjects with lymphatic pathology are usually amicrofilaremic, have enhanced blastogenic responses to filarial antigens and produce Th1 cytokines (IFN-γ and IL-2). IL-4 and IL-10 have potent negative immunoregulatory functions by which they suppress cell-mediated immune responses and favor persistence of the parasites (Hart et al., 1989; Martinez et al., 1990; Bodgan et al., 1991; De Waal Malefyt et al., 1991). It appears obvious that the deviation of the immune response towards a Th2 pattern controls inflammatory responses in microfilaremic asymptomatic patients (Nutman, 1995).

In the jird (Meriones unguiculatus) infected with B. pahangi, the implication of the immune response in the development of lymphatic granulomas has been well demonstrated. Presensitization and protective immunity results in increased severity of lymphatic lesions (Klei et al., 1982; Petit et al., 1993). Chronic microfilaremic jirds manifest a filarial specific state of hyporesponsiveness (Lammie and Katz, 1983) accompanied by decreased lymphatic lesion severity (Klei et al., 1981; Klei et al., 1990).
Measurement of cytokine mRNA from spleen and lymph nodes of
*B. pahangi*-infected jirds revealed IL-4 and IL-10 messages as
early as 28 days post infection (DPI), followed by a marked
increase of these cytokines at 56 and 150 DPI (Mai, 1996).
This pattern of cytokines mimics that described in
hyporesponsive filariae-infected human patients (King et al.,
1993; Maizels et al., 1995).

The MAC is the main cellular component of the granuloma
and is often present covering the surface of filariae (Nelson
et al., 1976; Jeffers et al., 1987), (chapter 3) and other
nematodes (Soulsby, 1963; Jeska, 1969). Even though the MAC
is a major component in the inflammatory reactions to
multicellular parasites, its role in pathology and
immunomodulation during helminth infections has not been
studied extensively. The implication of T cell-mediated MAC
activation in the destruction of metazoan parasites has only
been amply investigated in schistosomiasis, where it has been
shown that MAC activation is important in resistance (Civil
et al., 1978; Mahmoud et al., 1979; James et al., 1982a;
James et al., 1982b). The prominent granulomatous
inflammation around schistosoma eggs characteristic of the
acute state of the disease is gradually downmodulated as the
infection progresses to the chronic phase. The role of the
MAC in schistosoma "immunomodulation" has been reviewed
(Stadecker, 1994). MACs from egg granulomas induce anergy in
Th1 lymphocytes (Stadecker, 1992; Stadecker et al., 1990) and
in Th-1 clones specific for egg antigens (Flores Villanueva et al., 1994). Granuloma MACs are also capable of downmodulating the granulomatous lesions in vivo (Flores Villanueva et al., 1994). Further, IL-10 was found to exert a potent downregulatory effect on antigen presenting cells. Culture of granuloma MACs in the presence of anti-IL-10 mAb resulted in upregulation of MHC class II molecules, and the costimulatory molecules B7-1 and B7-2 on granuloma MACs as well as in the ability to stimulate egg antigen-specific T cell responses (Stadecker, 1994).

Rat and mouse s have been shown to participate in cytotoxic reactions presumably mediated by antibody and/or complement against Brugia spp MF (Oxenham et al., 1984; Chandrashekar et al., 1985; Chandrashekar et al., 1986) and L3 (Chandrashekar et al., 1985) in vitro. Studies in the jird have demonstrated that B. pahangi infection results in increased capacity of MACs to phagocytize and kill Staphylococcus aureus (Jeffers et al., 1984).

The purpose of the experiments described in this chapter was to determine the level of jird MAC activation and TNF production following in vivo infection with B. pahangi. The effect of different parasite stages (female or male worms) on MAC function was also investigated.
MATERIAL AND METHODS:

Animals

Inbred, female, 6 to 8 week old jirds (*Meriones unguiculatus*) were obtained from Tumblebrook Farms (West Brookfield, MA). Inbred, female, Balb/c mice were kindly provided by Dr. J.L. Krahenbuhl and were originally obtained from Jackson Laboratories (Bar Harbor, Maine). Animals were maintained on standard rodent chow and water ad libitum. All jirds and mice used in these experiments were infected at 3 to 4 months of age.

*In vivo* infection with BCG

Briefly, one group of 24 jirds and one group of 24 mice were inoculated intradermally (ID) with $3 \times 10^6$ colony-forming units of living *Mycobacterium bovis*, strain bacillus Calmette-Guerin (BCG) (kindly provided by Dr. J.L. Krahenbuhl). 24 and 48 hours before euthanasia BCG immunized animals were inoculated intraperitoneally (IP) with 50 ug of purified protein derivative of the tubercle bacillus (PPD) (Parke-Davis, Rochester, MI) dissolved in 0.5 ml of phosphate buffered saline (PBS). The BCG-PPD immunization protocol has previously been described (Ruco and Meltzer, 1977). Control animals, 24 per group, were inoculated ID with 0.05 ml of PBS and IP with 0.5 ml of PBS. Necropsies were performed at 15, 28 and 42 days post infection (DPI) with BCG.

*Brugia* Parasites

The *B. pahangi* life cycle was maintained in *Aedes aegypti* and jirds as previously described (Klei et al.,
Male and female adult *B. pahangi* were aseptically collected from the peritoneal cavities of jirds with chronic patent IP infections. The worms were washed in RPMI-1640 medium (GIBCO, Grand Island, NY) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) prior to being transferred to 3 ml syringes. Single sex implantations of 10 female or 10 male worms into the peritoneal cavity of jirds were done using 16 gauge needles. Control animals were inoculated IP with RPMI medium. Necropsies were performed at 15, 50 to 56, and 135 DPI.

**Macrophage culture**

Peritoneal cells were aseptically collected from the peritoneal cavities of jirds and mice in PBS containing 10 units/ml of heparin (Sigma). Peritoneal cells were washed once at 250 x g for 10 min and transferred to RPMI-1640 supplemented with antibiotics (described above), HEPES buffer (25 mM), 2 x 10^{-5} M 2-mercaptoethanol, L-glutamine (2 mM) and 10% heat inactivated fetal bovine serum (FBS). Cells were prepared on LUX cover slips (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, IL) in 24-well tissue culture plates (GIBCO, Grand Island, NY) at 3 x 10^6 cells/well and were incubated at 37°C. After 4 to 6 hours of culture non-adherent cells were washed off by lavage with PBS. MAC monolayers on the coverslips were used in the bioassays for *Toxoplasma* killing and for tumor necrosis factor (TNF) and Nitric oxide (NO) production.
Spleen cell culture and collection of macrophage activating factors

Macrophage activating factors (MAF) were collected from cultures of spleen cells from BCG-PPD inoculated animals or from untreated animals.

Single cell suspensions were obtained from spleens of BCG-PPD inoculated and untreated animals as described previously (Klei et al., 1990). Briefly, spleens were removed, placed on a nylon mesh screen and partially minced with scissors. Using a 10 cc disposable syringe plunger, the spleen pieces were pressed thru the screen into a petri dish containing complete medium. The spleen cell suspension was concentrated by centrifugation. Red blood cells were removed by suspending the cell pellet in prewarmed NH₄Cl solution (Sigma) (approximately 3 ml per spleen) and gently shaking the suspension for 3 min in a 37°C water bath. Cells were washed once in complete medium (250 x g, 10 min) and cultured in 5% FBS, RPMI-1640 medium supplemented as indicated above.

To obtain BCG-PPD MAF, spleen cells were incubated at a concentration of 4 x 10⁶ cells/ml with 40 μg/ml of PPD in 25 cm² plastic tissue culture flasks (Costar, Cambridge, MA) (Nacy et al., 1985). After 48 and 72 hours, culture supernatants were collected, centrifuged at 10,000 x g for 10 min and stored at -20°C until use.

To obtain Con A MAF, spleen cells were incubated at a concentration of 1.5 x 10⁶ cells/ml with 1, 2 or 3 μg/ml of
Con A (Sigma) in 25 cm² plastic tissue culture flasks for 24, 48, 72, 96 or 120 hours. Culture supernatants were collected, centrifuged at 10,000 x g for 10 min and stored at -20°C until use. Previous work in our laboratory and by other investigators (Lammie and Katz, 1983) have demonstrated that the concentrations of Con A used at the different time intervals to obtain MAF induced spleen cell proliferation measured by tritiated thymidine incorporation.

Reagents

Bacterial lipopolysaccharide (LPS) from *Escherichia coli* 0111.B4 was used (Sigma). Recombinant murine IFN-γ was originally obtained from Genentech, Inc (South San Francisco, CA).

In vitro activation of macrophages

A combination of either BCG-PPD MAF, Con A MAF (1:1, 1:2 or 1:3 MAF/medium) or murine rIFN-γ (500 or 1000 U/ml) with 10 or 50 ng/ml of LPS were added to the MAC monolayers. MACs were cultured undisturbed overnight. Attempts to activate jird and mouse MACs in vitro were repeated at least three times for each treatment.

Toxoplasma killing assay

Tachyzoites of *Toxoplasma gondii* RH strain were harvested from the peritoneal cavities of Balb/c mice 2 days after infection and purified by filtration through 3 μm polycarbonate membranes (Nucleopore Corporation, Pleasanton, CA) as described previously (Wilson et al., 1980; Sibley and
Krahenbuhl, 1988). MAC monolayers from controls and from animals infected with BCG-PPD or B. pahangi were challenged with 1.5 x 10^6 freshly harvested T. gondii cells. 1 hour later extracellular T. gondii cells were rinsed off and MAC monolayers were returned to culture. Microbicidal activity was assessed after 20 h of culture. Coverslips were fixed and stained with Hema 3 (Curtin Matheson Scientific, Inc, Houston, TX). The number of intracellular Toxoplasma were counted in 100 MACs per coverslip. Triplicate samples were done for each treatment.

**Tumor Necrosis Factor production**

Levels of TNF-α-like activity were determined in supernatants of MAC cultures by a modified L929 fibroblast cell lytic assay (Agarrwal et al., 1985; Sibley et al., 1991). MAC monolayers were prepared as described above. MACs were either stimulated with 50 μg/ml of LPS or unstimulated. Supernatants were collected after 4 hours, centrifuged at 10,000 x g for 10 mn and stored at -70°C until use. Duplicate samples were serially diluted three-fold in 96 well, flat bottoned tissue culture plates (Costar, Cambridge, MA). L929 cells (kindly provided by Dr. J.L. Krahenbuhl) were harvested in active, log-phase growth. L929 cell suspension containing 2 μg/ml of actinomycin D (Sigma) was prepared at a concentration of 3 x 10^6 cells/ml. 100 μl/well of cell suspension was added to each well and plates were incubated at 37°C. After 24 hours medium was emptied
from plates and 0.1% of crystal violet was added. Plates were incubated at room temperature for 10 min. Plates were rinsed with water after emptying the crystal violet (Sigma). 100 μl of 1% sodium dodecyl sulfate (SDS) (Sigma) were added in each well. Absorbance was read at 570 nm. Concentrations of TNF were calculated in units defined as the reciprocal dilution which yields 50% lysis of L929 cells.

Measurement of Nitrite production

Levels of nitrite (NO\textsubscript{2}^-) in MAC supernatants were determined spectrophotometrically at 540 nm following reaction with the Griess reagent (Adams et al., 1990; Drapier and Hibbs, 1988). NO\textsubscript{2}^- is the stable end product of nonenzymatic degradation of nitric oxide (NO). NO\textsubscript{2}^- levels were measured in supernatants from MAC monolayers treated in vitro with either IFN-γ or Con A MAF and LPS as second signal or only with LPS. NO\textsubscript{2}^- production was also determined in cultured MAC from BCG-PPD immunized animals. NO\textsubscript{2}^- concentration was calculated from a NaN\textsubscript{2} standard curve and results are expressed in μM (micromoles).

Statistical Analysis

Results were analysed statistically with a comparative analysis of variance using the Tukey’s Studentized Range test.
RESULTS

Macrophage activation after in vivo infection of jirds and mice with BCG.

Prior to experimental filarial infection, the activation of jird MACs was characterized by in vitro and in vivo methods. All attempts to activate jird MACs in vitro with supernatants from Con A stimulated spleen cultures or with murine rIFN-γ as a first signal, followed by LPS as second signal failed (Table 4.1.). However, parallel experiments with murine MACs treated with mouse Con A MAF or murine rIFN-γ consistently yielded positive results (Table 4.1.). Furthermore, mouse Con A MAF or murine rIFN-γ did not restrict Toxoplasma growth in jird MACs. Jird Con A MAF, however, conferred toxoplasmacidal activity to mouse MACs. This activity was significantly less than that induced by INF-γ or mouse MAF (P < 0.05).

Alternatively, we chose to activate MACs in vivo with BCG, as previously demonstrated in the murine system (Ruco and Meltzer, 1977). Results showed that MACs recovered from BCG-PPD treated mice and jirds restricted the intracellular growth of Toxoplasma cells when compared to control MACs at the three time periods tested (15, 28, 42 DPI) (P < 0.05). Microbicidal activity of BCG-PPD treated peritoneal MACs was manifested by a decrease in the percentage of Toxoplasma-infected MACs (Fig. 4.1. and 4.4.), decrease in the number of Toxoplasma cells per infected MAC (Fig. 4.2. and 4.5.) and
Table 4.1. Effects of Con A MAF and murine rIFN-γ on the toxoplasmacidal activity of jird and mouse macrophages*. LPS was added as a second signal.

<table>
<thead>
<tr>
<th></th>
<th>JIRD MACROPHAGES</th>
<th>MOUSE MACROPHAGES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control(^c)</td>
<td>Murine rIFN-γ + LPS</td>
</tr>
<tr>
<td>% INFECTED</td>
<td>22.7 ± 7.13</td>
<td>17.33 ± 0.94</td>
</tr>
<tr>
<td>MACROPHAGES</td>
<td></td>
<td></td>
</tr>
<tr>
<td># TOXOPLASMA/INF</td>
<td>3.18</td>
<td>3.35</td>
</tr>
<tr>
<td>MACS</td>
<td></td>
<td></td>
</tr>
<tr>
<td># TOXOPLASMA/</td>
<td>72 ± 22.6</td>
<td>58 ± 10.98</td>
</tr>
<tr>
<td>100 MACS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control(^c)</td>
<td>Murine rIFN-γ + LPS</td>
</tr>
<tr>
<td>% INFECTED</td>
<td>15.57 ± 0.94</td>
<td>3.67 ± 2.05(^*)</td>
</tr>
<tr>
<td>MACROPHAGES</td>
<td></td>
<td></td>
</tr>
<tr>
<td># TOXOPLASMA/INF</td>
<td>6.64</td>
<td>1.27(^*)</td>
</tr>
<tr>
<td>MACS</td>
<td></td>
<td></td>
</tr>
<tr>
<td># TOXOPLASMA/</td>
<td>104 ± 15.77</td>
<td>4.67 ± 2.86(^*)</td>
</tr>
<tr>
<td>100 MACS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Results are expressed as mean ± standard deviation.

\(^a\) Indicates a significant difference (P < 0.05) from the controls.

\(^c\) Peritoneal macrophages from control uninfected animals that had no in vitro treatment.
Figure 4.1. Effect of BCG-PPD immunization on jird macrophage activation measured by the percentage of Toxoplasma-infected macrophages. Days postinfection indicate days after ID BCG inoculation. One and two days before collection of peritoneal macrophages animals were boosted IP with PPD. Macrophages from BCG-PPD immunized jirds were compared with control uninfected jirds. Bars represent the standard deviation of the mean.
Figure 4.2. Effect of BCG-PPD immunization on jird macrophage activation measured by the mean number of Toxoplasma per infected macrophage. Number of Toxoplasma per infected macrophage was calculated dividing total number of organisms in 100 macrophages by the percentage of infected macrophages.
Figure 4.3. Effect of BCG-PPD immunization on jird macrophage activation measured by the mean number of Toxoplasma per 100 macrophages.
Figure 4.4. Effect of BCG-PPD immunization on mouse macrophage activation measured by the percentage of Toxoplasma-infected macrophages. Days postinfection indicate days after BCG inoculation. Effect of in vitro murine rIFN-γ and LPS treatment on macrophage activation is also shown. Bars represent the standard deviation of the mean.
Figure 4.5. Effect of BCG-PPD immunization on mouse macrophage activation measured by the mean number of *Toxoplasma* per infected macrophage.
Figure 4.6. Effect of BCG-PPD immunization on mouse macrophage activation measured by the mean number of Toxoplasma per 100 macrophages.
decrease in the number of Toxoplasma per 100 MACs (Fig. 4.3. and 4.6.). Toxoplasmacidal activity did not vary significantly in mice or jirds throughout the infection. Jird MACs showed a lower capacity to restrict Toxoplasma growth than mouse MACs.

**Effect of BCG-PPD MAF on murine and jird macrophage activation.**

The addition of murine BCG-PPD MAF to MAC monolayers from control mice resulted in marked increase of microbicidal activity measured via Toxoplasma killing (Table 4.2.). However, jird BCG-PPD MAF did not restrict the growth of Toxoplasma in MACs from control jirds.

**Nitrite production**

NO$_2^-$ release was not detected in any of supernatants from treated or non-treated jird MAC cultures treated with Con A MAF or IFN-γ plus LPS or LPS alone. However, parallel experiments using murine MACs resulted in NO$_2^-$ production as previously described (Ding, 1988). The greatest NO$_2^-$ production was observed in murine MACs treated with a combination of IFN-γ and LPS ($P < 0.05$). Increase of NO$_2^-$ production occurred over time in LPS-treated murine MACs (Table 4.3.). NO production above control levels was not found in MACs from BCG-PPD immunized jirds (Table 4.4.).

**Macrophage activation after intraperitoneal infection of jirds with adult female or male *B. pahangi.***

Toxoplasmacidal activity was measured in peritoneal MACs from jirds with intraperitoneal infections with either adult
Table 4.2. Effects of BCG-PPD MAF on the Toxoplasmacidal activity of jird and mouse macrophages*. LPS was added as a second signal. BCG-PPD MAF from animals with 15, 28 and 42 days of BCG infection were used.

<table>
<thead>
<tr>
<th>JIRD MACROPHAGES</th>
<th></th>
<th>15 DPI BCG MAF + LPS^a</th>
<th></th>
<th>28 DPI BCG MAF + LPS^b</th>
<th></th>
<th>42 DPI BCG MAF + LPS^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>% INFECTED MACROPHAGES</td>
<td>Control^a</td>
<td>28.3 ± 0.47</td>
<td>21 ± 0.82</td>
<td>15.7 ± 1.25</td>
<td>15.5 ± 0.5</td>
<td>12.7 ± 1.69</td>
</tr>
<tr>
<td># TOXOPLASMA/INF MACs</td>
<td>4.91</td>
<td>4.03</td>
<td>4.29</td>
<td>4.45</td>
<td>3.87</td>
<td></td>
</tr>
<tr>
<td># TOXOPLASMA/100 MACs</td>
<td>139 ± 5.43</td>
<td>84.7 ± 14</td>
<td>67.3 ± 3.4</td>
<td>69 ± 7</td>
<td>49 ± 8.64</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MOUSE MACROPHAGES</th>
<th></th>
<th>15 DPI BCG MAF + LPS^a</th>
<th></th>
<th>28 DPI BCG MAF + LPS^b</th>
<th></th>
<th>IFN-γ + LPS^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>% INFECTED MACROPHAGES</td>
<td>Control^a</td>
<td>31.3 ± 2.49</td>
<td>7 ± 5^c</td>
<td>13.3 ± 1.7</td>
<td>1 ± 0^c</td>
<td>1 ± 0^c</td>
</tr>
<tr>
<td># TOXOPLASMA/INF MACs</td>
<td>6.5</td>
<td>1.28^c</td>
<td>8.5</td>
<td>1^c</td>
<td>1^c</td>
<td></td>
</tr>
<tr>
<td># TOXOPLASMA/100 MACs</td>
<td>204 ± 39</td>
<td>9 ± 7^c</td>
<td>146.7 ± 18</td>
<td>1 ± 0^c</td>
<td>1 ± 0^c</td>
<td></td>
</tr>
</tbody>
</table>

* Results are expressed as mean ± standard deviation.
^a,b Treatment groups with the same letter were done at the same time.
^c Indicates a significant difference (P < 0.05) from the controls.
Table 4.3. Nitric oxide production by murine and jird macrophages*. Results are expressed as μM of NO₂⁻. Note the absence of NO₂⁻ release from jird macrophages. Maximum NO₂⁻ production is present in murine macrophages treated with IFN-γ and LPS.

<table>
<thead>
<tr>
<th>HOURS OF CULTURE</th>
<th>JIRD MACROPHAGES</th>
<th>MOUSE MACROPHAGES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No treatment</td>
<td></td>
</tr>
<tr>
<td>4 hours</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>24 hours</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>LPS treatment</td>
<td></td>
</tr>
<tr>
<td>4 hours</td>
<td>0</td>
<td>1.49 ± 2.11</td>
</tr>
<tr>
<td>12 hours</td>
<td>0</td>
<td>12.96 ± 0.57*</td>
</tr>
<tr>
<td>24 hours</td>
<td>0</td>
<td>39.92 ± 2.25*</td>
</tr>
<tr>
<td></td>
<td>IFN-γ + LPS</td>
<td></td>
</tr>
<tr>
<td>20 hours</td>
<td>0</td>
<td>52.46 ± 1.31*</td>
</tr>
<tr>
<td></td>
<td>Jird Con A MAF + LPS</td>
<td></td>
</tr>
<tr>
<td>20 hours</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td></td>
</tr>
<tr>
<td>0 hours</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Results are expressed as mean ± standard deviation.

* Indicates a significant difference (P < 0.05) from samples with no treatment.

ND Not determined.
Table 4.4. Nitric oxide production by macrophages from jirds and mice immunized with BCG-PPD*. Results are expressed as $\mu$M of NO$_2^-$. 

<table>
<thead>
<tr>
<th>DAYS POST INFECTION$^a$</th>
<th>TX</th>
<th>ITX</th>
<th>MOUSE MACROPHAGES</th>
<th>JIRD MACROPHAGES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CONTROL$^c$</td>
<td>BCG-PPD$^b$</td>
</tr>
<tr>
<td>15 DPI$^a$</td>
<td>0</td>
<td>4h</td>
<td>1.29 ± 0.51</td>
<td>2 ± 0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LPS</td>
<td>1.5 ± 0.39</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>12h</td>
<td>ND</td>
<td>13.4 ± 14</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td></td>
<td>5.5 ± 0.8</td>
<td>22.7 ± 6.6</td>
</tr>
<tr>
<td>28 DPI</td>
<td>0</td>
<td>4h</td>
<td>0.15 ± 0.22</td>
<td>1.16 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LPS</td>
<td>ND</td>
<td>3.33 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>12h</td>
<td>ND</td>
<td>0.17 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td></td>
<td>ND</td>
<td>14.6 ± 4.4</td>
</tr>
<tr>
<td>42 DPI</td>
<td>0</td>
<td>4h</td>
<td>0.32 ± 0.45</td>
<td>4.6 ± 4.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LPS</td>
<td>0.63 ± 0.6</td>
<td>4.7 ± 6.7</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>12h</td>
<td>ND</td>
<td>14.3 ± 13.9</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td></td>
<td>ND</td>
<td>34.8 ± 20.8</td>
</tr>
</tbody>
</table>

* Results are expressed as mean ± standard deviation.

$^a$ Number of days post infection (DPI) with BCG.

$^b$ Animals were infected with BCG and shortly before euthanasia they were boosted IP with PPD.

$^c$ Uninfected animals.

TX Macrophages were cultured with LPS or with no LPS.

ITX Indicates hours of culture with LPS or with no LPS.

ND Not determined
female or male *B. pahangi*, and was compared to that of MACs from uninfected jirds or from BCG-PPD immunized jirds. MACs from jirds infected with adult female or male *B. pahangi* at 15 DPI restricted the growth of *Toxoplasma* cells in a manner similar to that of MACs from BCG-PPD immunized jirds. Percentage of infected MACs, number of *Toxoplasma* per infected MAC and total number of *Toxoplasma* per 100 MACs at 15 DPI were decreased with respect to control MACs (*P < 0.05*) (Fig. 4.7., 4.8. and 4.9.). MACs from female or male worm infections at 50 or 135 DPI showed percentage of infected MACs, number of mean *Toxoplasma* per infected MAC and total number of *Toxoplasma* per 100 MACs significantly higher than MACs from BCG-PPD immunized jirds and not significantly different from uninfected controls (*P < 0.05*) (Fig. 4.7., 4.8. and 4.9.).

**TNF-like production by peritoneal macrophages from jirds infected with adult female or male *B. pahangi*.**

Spontaneous release of TNF above controls occurred erratically in MACs from female and male worm-inoculated jirds throughout infection. Release of TNF from LPS-stimulated MACs was increased with respect uninfected controls at 15, 56 and 135 DPI in the female worm infection, and at 15 and 56 DPI in the male worm infection (Table 4.5.). In both female and male worm infections the LPS-stimulated TNF production peaked at 56 DPI and decreased markedly at 135 DPI.
Figure 4.7. Macrophage activation in jirds inoculated IP with female or male B. pahangi measured by the percentage of Toxoplasma-infected macrophages. The toxoplasmacidal activity of macrophages from jirds infected with female or male worms at 15 DPI was similar to that of macrophages from BCG-PPD immunized jirds. Bars represent standard deviation of the mean.
Figure 4.8. Macrophage activation in jirds inoculated IP with female or male \textit{B. pahangi} measured by the number of \textit{Toxoplasma} per infected macrophage. Toxoplasmacidal activity of macrophages from uninfected or BCG-PPD infected jirds is also shown.
Figure 4.9. Macrophage activation in jirds inoculated IP with female or male *B. pahangi* measured by the total number of *Toxoplasma* per 100 macrophages. Bars represent standard deviation of the mean. Toxoplasmacidal activity of uninfected or BCG-PPD infected jirds is also shown.
Table 4.5. Tumor necrosis factor-like production by peritoneal macrophages from jirds with female or male *B. pahangi*. Macrophage monolayers were nontreated (no LPS) or stimulated with 50 ng of LPS for 4 hours. TNF production was quantitated with the L929 cell line and expressed as units/ml. Note the marked increase of LPS-induced TNF that occurred in female and male worm infections at 15 and 56 DPI when compared with controls. Chronic worm infection resulted in prominent downregulation of TNF release.

<table>
<thead>
<tr>
<th></th>
<th>15 DPI</th>
<th>56 DPI</th>
<th>135 DPI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No LPS</td>
<td>LPS</td>
<td>No LPS</td>
</tr>
<tr>
<td>FEMALE WORM INFECTION</td>
<td>12.3 ± .2</td>
<td>95 ± 73.8</td>
<td>0.9 ± 1.4</td>
</tr>
<tr>
<td>MALE WORM INFECTION</td>
<td>3.6 ± 0.8</td>
<td>24 ± 2.5</td>
<td>3.2 ± 0.6</td>
</tr>
<tr>
<td>CONTROL</td>
<td>2.4 ± .04</td>
<td>8.7 ± 0</td>
<td>1.2 ± 0.9</td>
</tr>
</tbody>
</table>

* Results are expressed as mean ± standard deviation.

\( c \) Uninfected controls

DPI Days post infection with *B. pahangi*
DISCUSSION

Most MAC activation systems consist of IFN-γ as a first signal and a second signal represented by either the endogenous production of TNF-α (Green et al., 1990a; Sibley et al., 1991; Langermans et al., 1992) or by an exogenous factor (such as LPS) that induces TNF-α production (Green et al., 1992). MAC activation in the murine system has been exaustively studied. However, extrapolation to other species has not always been successful (Douvas et al., 1986; Rook et al., 1986; Toba et al., 1989; Krahenbuhl and Adams, 1994). Activation of jird MACs was not accomplished using standard in vitro methods routinely employed in the murine and other systems. Further, it was not possible to detect jird MAF in cultures of spleen cells stimulated with Con A or PPD obtained from normal or BCG-PPD immunized jirds, respectively. Parallel in vitro experiments in mice resulted in MAC activation indicating that these methods and reagents are effective in our laboratory. However, in vivo BCG-PPD immunization activated jird and murine MACs to kill Toxoplasma cells.

The reasons why jird MACs could not be activated in vitro to kill Toxoplasma via MAF are at this moment uncertain, but may be related to the inability of the jird to express significant amounts of IFN-γ. Jird IFN-γ mRNA was not detected in lymph nodes and spleens from uninfected or B.
pahangi-infected jirds and IFN-γ gene expression in Con A stimulated spleen cells was extremely low (Mai, 1996). However, this does not explain why jird MACs can be activated in vivo but not in vitro. It is possible that jird IFN-γ or other equivalent jird MAF becomes inactivated easily under certain conditions in vitro or that unknown inhibiting substances in culture conditions have specific effects on jird MAF. Human MACs treated with combinations of IFN-γ plus TNF-α or LPS are unable to decrease survival of Mycobacterium leprae (Krahenbuhl and Adams, 1994) or other mycobacteria (Douvas et al., 1986; Rook et al., 1986; Toba et al., 1989) as compared to mouse MACs (Krahenbuhl and Adams, 1994; Alfes et al., 1985; Rook et al., 1986). However, similar treatment of human MACs in vitro can accomplish killing of other different intracellular organisms (Murray and Cartelli, 1983; Hoover et al., 1985; Douvas et al., 1986). These differences in cell response between and within animal species indicate that the signaling networks that lead to MAC activation may be more complex than was first believed. Furthermore, the fact that the NO pathway cannot be demonstrated consistently in human MACs (review in Denis, 1994) has also raised doubts about the concept of MAC activation defined in the murine system. The NO pathway has been implicated as primary effector mechanism mediating cytotoxicity of activated MACs (Nathan and Hibbs, 1991). NO production was not found in cultures of LPS-stimulated and non-stimulated, peritoneal
MACs from control or BCG-PPD immunized jirds or in jird MAC monolayers treated with a combination of Con A MAF and LPS. However, parallel experiments with murine MACs demonstrated NO generation as previously reported by other investigators (Hibbs et al., 1987; James and Glaven, 1989; Adams et al., 1990; Green et al., 1990b).

It is possible that the differences seen between mouse and jird MACs to become toxoplasmacidal are due to variations in susceptibility to this protozoan. Results from in vivo BCG-PPD immunization suggest that mouse MACs have a higher capacity to restrict *Toxoplasma* growth than jird MACs. Other authors have found that although both mice and jirds appear to be equally susceptible to the highly virulent RH strain of *Toxoplasma*, the jird dies or develops severe clinical illness when infected with moderate or low virulence strains. Whereas in the mouse these strains induce a chronic latent infection (Suzuki and Tsunematsu, 1974) that results in the presence of highly activated MACs (Ruskin and Remington, 1968). Interestingly, humans and rats have a remarkable resistance to *Toxoplasma* infection (Lainson, 1955). Normal human and rat alveolar MACs can kill *Toxoplasma* *in vitro* and this microbial activity is oxygen-independent (Catterall et al., 1986) suggesting that the particular resistance of these species is due to the presence of a powerful non-oxidative antimicrobial mechanism that could innately be turned on. The requirements to trigger microbicidal mechanisms may vary
widely with animal species and specific pathogens which would explain the differences we found in the mouse and jird systems of MAC activation.

Peritoneal MACs from jirds infected with female or male *B. pahangi* were toxoplasmacidal at 15 DPI, but not at 50 DPI and 135 DPI. MAC activation in early *B. pahangi* infection coincided with the peak in granulomatous reactivity around parasite antigen-coated beads reported in chapter 2. Lack of toxoplasmacidal activity corresponded to downregulation of the pulmonary granulomatous response. No difference in MAC microbicidal activity was demonstrated between female or male *B. pahangi* infections, indicating that the presence of MF was not required for MAC deactivation. Similarly, data from chapter 2 indicate that downregulation of the PGRN is not dependent on the presence of MF. The increase of Th2 cytokine messages, mRNA IL-4 and IL-10, in cells from spleen and lymph nodes of subcutaneously *B. pahangi*-infected jirds (Mai, 1996) occurs at the same time periods than MAC deactivation in our experiments. A state of filarial immune hyporesponsiveness with absence of marked inflammation has also been associated with a shift to the Th2 cell phenotype in humans (King et al., 1993; Maizels et al., 1995). The lack of toxoplasmacidal activity of MACs in chronically *B. pahangi*-infected jirds may be related to the presence of Th2 cytokines that deactivate MACs, such as IL-4 (Lehn et al., 1989; Ho et al., 1992) and IL-10 (De Waal Malefyt et al.,
These cytokines exert a negative effect on proinflammatory molecules which would explain the depressed PGRN found in our experiments.

Female worm infection resulted in high accumulation of peritoneal MACs presumably due to the continuous release of MF that acted as a potent inflammatory stimulus (chapter 2). Interestingly, these MACs were not activated to kill Toxoplasma and rarely formed granulomas. As it has shown in diverse intracellular organisms (review in Reiner, 1994), the interaction MAC-parasite causes defects on the MAC effector functions that may result in suppressive effects on other immune cells. For instance, MACs can be induced to secrete IL-10, TGF-β and prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) that downregulate cell-mediated immunity and may drive the immune response to a Th2 phenotype. Molecules from filarial nematodes that may exert a direct effect of MAC function have not been well studied. However, MF have been shown to release PGE\textsubscript{2} (Liu et al., 1992). This inflammatory molecule has been demonstrated to be a potent immune modulator suppressing MAC (Taffet and Russell, 1980; Snyder et al., 1982) and lymphocyte functions (Ellner and Spagnuold, 1986).

Previous studies demonstrated that MACs from B. pahangi-infected jirds with chronic infections were activated to kill S. aureus (Jeffers et al., 1984). These results may differ from the current data because the stage to initiate infection
were L3, which in the chronic phase resulted in greatest MAC accumulation with characteristic granuloma formation (Jeffers et al., 1987). On the other hand, the immunological requirements to kill *Toxoplasma* cells may vary from those to kill *S.aureus*. Microbicidal activity to kill *S.aureus* of MACs from L3 *B.pahangi*-infected jirds was similar to that of thioglicolate-elicited control MACs. However, attempts to obtain toxoplamacidal activity in thioglicolate-elicited MACs failed (data not shown). Killing of *Toxoplasma* organisms probably requires different immune-mediated signals than killing of facultative organisms such as *S. aureus*. Other investigators, however, have found variations in the ability of MACs to cope with organisms of more similar background. For instance, it has been found that susceptibility of *Leishmania* to be killed by presumably activated MACs varies markedly upon species and stages of the parasite (Murray and Cartelli, 1983; Hoover et al., 1985).

Increase in spontaneous and LPS-induced TNF production above that of controls occurred at 15 DPI in both female and male worm infections corresponding to MAC activation. LPS-induced TNF production peaked at 56 DPI and decrease markedly at 135 DPI in both female and male infections. The peak in TNF production occurred at the moment MACs were accumulating in large numbers, especially in the female infection; and may be related to the potent chemotactic function of this cytokine. The subsequent decrease could have been induced by
similar factors that caused MAC deactivation. It has been demonstrated that deactivating cytokines such as IL-10 and IL-4 inhibit production of TNF-α (Hart et al., 1989; De Waal Malefyt et al., 1991). These cytokines may be more abundant or may exert a more intense downregulatory effect as the infection progresses to the chronic time period. IL-4 and IL-10 mRNA in *B. pahangi*-infected jirds are highest at 150 DPI (Mai, 1996).

Further work is needed in the jird model to discern the factors and mechanisms involved in the downmodulation of the inflammatory and immune responses to filariae. We demonstrated that the parasite-specific hyporesponsive state defined in the jird infected with *B. pahangi* is associated with a defect in MAC function that is manifested as an incapacity to kill *Toxoplasma* and to produce TNF-α. The MAC is recruited locally in large numbers in response to filariae infection and may prove to be a key effector cell implicated in the immunoregulatory mechanisms that determine the disease outcomes in human filariasis.
CHAPTER 5. CONCLUSIONS

Lymphatic filariasis is a human parasitic disease with a spectrum of clinical conditions that reflect the intensity and pattern of immune responses to the parasite (King and Nutman, 1991). The two principal poles of the clinical states are asymptomatic microfilaremia and amicrofilaremic chronic pathology.

The jird-\textit{B. pahangi} model has been extensively used to study immunopathogenesis of filariae and has been proved to be a suitable experimental system to investigate factors implicated in filarial immunomodulation and pathology. The jird intraperitoneally infected with different stages of \textit{B. pahangi} was used to further characterize the stage specificity of the inflammatory response and the role of the MAC in the downmodulation of this response.

The second chapter addressed the effect of different \textit{B. pahangi} developmental stages as well as dead worms, and different parasite numbers on the downmodulation of the systemic inflammatory response. Results indicated that viable worms are required to induce downregulation of the inflammatory response, and this downregulation occurred in the absence of MF. Inoculation of male, female, L4, L3, and MF resulted in decreased PGRN as the infection progressed to the chronic period. Higher female worm burdens induced a significantly greater and more rapid PGRN decrease than low
burdens. PGRN decrease induced by adult worms was greater than that induced by MF. Therefore, data also indicated that parasite burden and probably chronicity effected the state of filarial hyporesponsiveness.

Experiments described in chapter 3 were conducted to better discern the role of developing larvae in the downmodulation of the PGRN. Jirds were inoculated with normal L3 or with L3 irradiated with increasing levels of gamma radiation. Gamma radiation inhibited the growth and decreased the survival of *B. pahangi* as previously reported (Oothuman et al., 1978; Devaney et al., 1993). Decrease in parasite survival and worm lengths was inversely related to the levels of irradiation as observed by Oothuman et al. (1978). Some parasites irradiated with the lowest radiation doses (15 and 25 krads) developed into adult female worms. Male worms were not found and females were not gravid. Downmodulation of the PGRN did not occur in jirds that received L3 irradiated with more than 15 krads. However, normal L3 and L3 irradiated with 15 krads induced significant PGRN decrease at the chronic time period. These results support the importance of the adult stage in the downregulation of the systemic inflammatory response and prove again that MF are not needed to induce this downregulation. It is not clear whether L4 are important in the downmodulation of the PGRN. However, the fact that PGRN decrease occurred at 28 DPI when the molt to adults had just
occurred, suggested that L4 do induce some level of downregulation. The absence of PGRN downregulation in jirds infected with L3 exposed to radiation doses higher than 15 krad may be explained by the low total parasite burden, the absence or minimal numbers of adult worms, or by the effect of high radiation on the release of specific parasite products.

Experiments described in chapter 2 and 3 showed that the peritoneal inflammatory response, measured by MAC numbers, was greatest in jirds with persistent intraperitoneal MF burdens. This indicated that the release of MF constitute a potent inflammatory stimulus in situations where compartmentalization may occur, and that this developmental stage may contribute to the lymphatic pathology seen in elephantiasis cases. Formation of peritoneal granulomas in jirds with normal L3 infections occurred more frequently at the chronic time period when MF were produced. Peritoneal granulomas were found less often in infections with female worms. This may be explained because MF numbers were lower in these latter infections. Alternatively, the early presence of adult worms may prevent formation of peritoneal granulomas by downmodulatory mechanisms similar to those that decreased the PGRN, but still allowed the recruitment of considerably number of MACs due to the presence of parasite products and/or MF. Infection with irradiated parasites resulted in frequent formation of peritoneal granulomas at 7
and 14 DPI, when compared to the paucity of granulomas at these time points following infections with normal L3. Dead and damaged larvae were probably more abundant shortly after infection with irradiated L3 and presumably were more capable of inducing an inflammatory reaction. Irradiated parasites that survived probably were not damaged and less effective at inducing local inflammation.

Chapter 4 described the studies on MAC function in jirds infected IP with female or male *B. pahangi*. The microbicidal activity against *Toxoplasma*, as well as NO and TNF production were used as indicators of MAC activation.

Prior to experimental filarial infection, jird MAC activation was characterized by comparing it to mouse MACs. Attempts to confer toxoplasmacidal activity to MACs *in vitro* with a combination of murine rIFN-γ, Con A MAF or BCG-PPD MAF and LPS succeeded in the mouse but failed in the jird. *In vivo* immunization with BCG-PPD yielded positive results in both species. However, the toxoplasmacidal activity of mouse MACs appeared to be greater than that of jird MACs after BCG-PPD immunization, which may be related to a higher susceptibility of jirds to *Toxoplasma* as other studies have shown (Suzuki and Tsunematsu, 1974). NO production by jird MACs could not be demonstrated after *in vitro* or *in vivo* treatments to activate MACs.

MAC activation occurred in jirds infected with female or male *B. pahangi* at 15 DPI, but not at 50 and 135 DPI. TNF-
like production increased at 15 DPI in both, female and male worm infections corresponding to MAC activation. LPS-induced TNF production peaked at 56 DPI and decreased markedly at 135 DPI in both female and male worm infections. MAC deactivation and decrease of TNF levels coincided with the downregulation of the PGRN. These data indicate that the factors responsible for the absence of MAC activation and decrease of TNF levels observed during chronic Brugia infection may be related to the mechanisms that induce the downmodulation of the systemic inflammatory response.
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Carmen Nasarre, the daughter of Antonio Nasarre and Montserrat Azara, was born in Barcelona, Spain, on October 26, 1967. She obtained her Doctor of Veterinary Medicine degree from the Faculty of Veterinary Medicine, University of Zaragoza, Spain, in September, 1990. Her determination to pursue a career in Pathology brought her to Louisiana in August, 1991 by mediation of Dr. Jose Maria Blasco, Dr. Enright and Dr. Casey. She started her PhD program in the department of Veterinary Pathology where she completed a pathology residency. In June, 1993 she joined Dr. Klei’lab to work in lymphatic filariasis. She then became a PhD candidate in the department of Veterinary Microbiology and Parasitology.
Candidate: Carmen Nasarre

Major Field: Veterinary Medical Sciences

Title of Dissertation: Inflammatory Responses of the Jird to *Brugia pahangi*: Parasite Stage Specificity and Role of the Macrophage

Approved:

[Signature]
Major Professor and Chairman

[Signature]
Dean of the Graduate School

EXAMINING COMMITTEE:

[Signature]

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