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Specific hypo-responsive granulomatous tissue reactions in *Brugia pahangi*-infected jirds

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Summary

Two types of experiments were used to study the degree of tissue responsiveness which occurred in *Brugia pahangi*-infected jirds. In experiment 1, the severity of lymphatic lesions which developed following subcutaneous infection of jirds which had existing intraperitoneal infections was compared to the severity of lymphatic lesions that developed following subcutaneous infection of jirds without previous infections. In experiment 2, comparisons were made of the sizes of granulomas which formed in the lungs around cyanogen bromide-activated Sepharose 4B beads coupled with *B. pahangi* antigen, which were inoculated intravenously into jirds which: 1. had existing lymphatic infections; 2. were uninfected but sensitized with *B. pahangi* antigen; or 3. were nonsensitized and noninfected. Results of both experiments indicated that jirds with intraperitoneal or lymphatic infections of *B. pahangi* of 120 to 160 days duration were significantly less responsive to *B. pahangi* or *B. pahangi* antigens as measured by pathologic tissue reactions than were uninfected jirds. The sizes of granulomas which formed around beads coupled to *B. pahangi* antigen in noninfected and nonsensitized jirds suggest that this worm material may contain factors which nonspecifically enhance the granulomatous inflammatory reaction.

Key words: Brugia pahangi; jird; gerbil *Meriones unguiculatus;* granuloma; immunosuppression; filariasis.

Introduction

Filarial specific hypersensitivity reactions have been suggested to be responsible for the lymphatic lesions of filariasis for many years. More recent

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clinical and immunologic studies of human filariasis patients have led Ottesen (1980) to associate specific clinical conditions with different immunologic states found in these patients. These states range from hypersensitive individuals showing signs of tropical eosinophilia to asymptomatic-infected individuals who are hypo-responsive to filarial antigens. Experiments using the *Brugia pahangi*-jird model have demonstrated that jirds hypersensitized to *B. pahangi* antigens prior to infection develop more severe lymphatic pathology (Klei et al., 1981). These studies indicate that the immune response is associated with lymphatic pathology in this animal model of filariasis. The present paper reports on experiments which provide evidence for the existence of a hypo-responsive condition, in the *B. pahangi*-jird model, as measured by the degree of severity of granulomatous lesions.

Materials and methods

Experimental design

Two experiments were performed using different methods to quantitate inflammatory reactions in jirds to *B. pahangi* or *B. pahangi* antigens.

Experiment 1. Thirty-six male outbred mongolian jirds *(Meriones unguiculatus)* 8 to 10 weeks of age (Tumblebrook Farms, West Brookfield, MA, USA) were divided into 3 groups of 10 jirds each. Two groups received intraperitoneal (IP) inoculations of 100 *B. pahangi* infective larvae (L_3), and the third remained uninfected. One IP infected group and the uninfected group were subsequently infected subcutaneously (SQ) with 100 L_3 125 days following the IP inoculations. An additional fourth group of 6 jirds remained uninfected and served as controls for spontaneous reactions. Serum was collected 1 to 7 days prior to SQ inoculations and at necropsy for antibody quantitation. Quantitative microfilarial determinations were made at necropsy. Necropsies were performed 90 days following the SQ inoculations. The severity of lymphatic lesions observed grossly was compared between groups by the methods discussed below.

Experiment 2. Forty-five jirds similar to those used in experiment 1 were divided into 3 groups of 15 jirds each. Jirds in one group were infected SQ with 100 *B. pahangi* L_3 on day 0. Jirds in the second group were sensitized with 0.5 ml of Freund's complete adjuvant (FCA) by SQ and intramuscular inoculations 3× at 10 day intervals beginning on day 130. The concentration of antigen (Ag) was 300 μg/ml. The third group remained uninfected and unsensitized. Following these treatments each group was further divided into 3 groups of 5 jirds each. These 3 groups of jirds were inoculated intravenously (IV) with cyanogen bromide-activated Sepharose 4B beads coupled with one of 3 substances. The 3 substances were: 1. *B. pahangi*-Ag, 2. methylated bovine serum albumin (BSA), or 3. diethanolamine (DEA). Necropsies were performed on day 160. At necropsy, lungs containing beads were gently inflated by inoculation of 10% neutral buffered formalin into the trachea, and further fixed by emersion in formalin. Granulomatous reactions around coupled beads in the lung were measured, lymphatic lesion quantation, and worm recoveries were performed using methods described below. All jirds were bled at necropsy for serum and microfilarial quantitations.

Infection, worm recoveries and lymphatic lesion quantitation

These methods have been described in detail previously (Klei et al., 1981a; Ah et al., 1974) and will only be summarized below. *B. pahangi* L_3 were obtained from Dr. John W. McCall of University of Georgia through the U.S.–Japan Cooperative Medical Science Program of the U.S. National Institutes of Health. Jirds were infected SQ by inoculation of 100 L_3 into the left inguinal area, and

IP by inoculation of 100 L_3 directly into the abdominal cavity. Microfilarial levels were quantitated by counting microfilariae per 20 μl samples of blood which were smeared, dried and giemsa stained. Animals were examined at necropsy with the aid of a stereomicroscope and the numbers of worms recoverd, their locations in the lymphatic vessels and the lesions present were recorded.

Lymphatic lesion scores ranging from 0 to 4 were determined for each animal with 4 representing the most severe change. The criteria used as approximate guides for the determination of each individual score were as follows: 0, no change; 1, no lymph thrombi seen in lymphatic vessels, 10 to 20% of the length of the infected spermatic cord vessels dilated, and 0 to 2 visible subcapsular lymph thrombi in renal and/or lumbar lymph nodes; 2, 1 to 5 lymph thrombi seen, 30 to 50% of the length of the spermatic cord lymphatic vessels dilated; and 2 to 4 visible subcapsular lymph thrombi in the renal and/or lumbar lymph nodes; 3, 5 to 10 lymph thrombi seen in lymphatic vessels, 50 to 80% of the length of the spermatic cord lymphatic vessel dilated, and 4 to 6 visible subcapsular lymph thrombi in the renal and/or lumbar lymph nodes; 4>10 lymph thrombi seen in lymph vessels, >80% of the spermatic cord lymphatic vessels showing dilation and >6 visible subcapsular lymph thrombi or diffuse uncountable clusters of thrombi in the renal and/or lumbar lymph nodes.

Photographs at magnifications of 2 to 3× were made of the renal lymph nodes, lumbar lymphatics, and spermatic cord lymphatics. Estimates of lymphatic vessel size were made in experiment 1 using these photographs by the following technique. Photographic slides were projected using a photographic enlarger at a consistant magnification of 2× onto bonded drawing paper and traced. These drawings were cut out and the papers weighed. The mean weight of the drawings from 5 age matched uninfected jird spermatic cord lymphatics was used as a standard for normal lymphatic vessels. Percent increases in infected spermatic cord lymphatic vessels were then calculated as a percent increase in the weight of drawings of infected vessels compared to noninfected vessels. Similar photographic methods were used to compare renal lymph node sizes, by drawing the periphery of these lymph nodes. Methods used to determine lymphatic lesion score have been previously described (Klei et al., 1981a).

Cyanogen bromide-activated Sepharose 4B beads and lung granuloma measurements

Cyanogen bromide-activated Sepharose 4B beads (Pharmacia, Piscataway, NJ, USA) were sized by washing through a series of 4 siliconized screens with mesh sizes ranging from 80 to 325 mesh with 0.001 M HCL. Beads collected in this filtrate ranged in size from 10 to 50 μm. These beads were coupled with *B. pahangi*-Ag, BSA or DEA using the methods of Axen et al. (1967). Coupled beads were diluted to concentrations of 1.0×10^5 per ml and 0.1 ml of the bead mixtures were inoculated into the periorbital plexus. This type of inoculation resulted in the lodging of significant numbers of beads in the pulmonary capillaries. Previous unpublished observations in our laboratory on the kinetics of hypersensitivity granuloma formation to BSA coupled beads in BSA sensitized jirds indicated that granulomas were at a maximal size 3 days post inoculation. Thus, lungs containing beads were fixed 3 days post inoculation in this study. Five step sections at 200 μm intervals were taken from each lobe of the lung, and these were stained with hematoxylin and eosin by standard methods. Granulomas which could be visualized around the standard sized beads (approximately 40 μm) were drawn with the aid of a drawing tube and the diameters of the two axes were measured. Twenty-five granulomas were measured and the mean diameters were determined as quantitative indicators of reactivity. Granulomas were examined for qualitative differences by the single blind method.

Antigen and serologic methods

Soluble somatic Ag was prepared from male and female adult *B. pahangi* which were previously recovered from the peritoneal cavities of jirds. Following their collection these worms were washed 5× in physiologic saline and stored frozen at –90° C. Worms were later thawd, crushed with a Ten Broeck type tissue grinder in 0.1 M phosphate buffered saline pH 7.2 (PBS) and extracted into PBS at 4° C for 12 to 18 h. The resulting suspension was centrifuged at 5,000 *g* to remove particulate

material and stored in small aliquots at –90° C. Indirect hemagglutination assays were performed on sera from experiment 1 using techniques described by Herbert (1978).

Statistical tests

Granuloma sizes and worm recoveries were compared statistically using analysis of variance and Duncan's multiple range test for simultaneous comparison of multiple treatment means. Criteria used to quantitate lesion severity in the two groups of jirds receiving SQ inoculations were compared statistically using Student's t-test.

Results

Experiment 1

Parasitologic findings of experiment 1 are shown in Table 1. The percentage of adult worms recovered from jirds receiving intraperitoneal inoculations alone were lower than that of the other two groups. Worms were not found in the lymphatic vessels of any animals in this group. Jirds in the 2 groups receiving SQ inoculations were not significantly different in total percent recoveries of adult worms or in the numbers of adult worms recovered from any lymphatic site. The percentage of patent infections that developed and the mean numbers of circulating microfilariae were higher in the group which received IP inoculations prior to the SQ inoculation.

Mean indirect hemagglutination antibody titers against *B. pahangi*-Ag were 1:512 in both IP inoculated groups prior to SQ inoculations and 1:2 in the uninfected groups at this time. Antibody levels in the two IP inoculated groups

Table 1. Summary of mean worm recovery, worm locations and microfilarial levels at necropsy in experiment 1

	Treatment		
	Intraperitoneal inoculation	Subcutaneous inoculation	Intraperitoneal and subcutaneous inoculations
Percent worms recovered	8.3 B**	18.4 A	21.7 A
No. of worms in lymphatics	0	13.4 A	19.5 A
No. of worms per spermatic cord lymphatics	0	4.9 A	6.4 A
No. of worms in peritoneal cavity	7.9 A	2.7 B	14.6 A
No. of worms in nonperitoneal nonlymphatic	0.4 B	2.3 A	7.2 A
Percent patent infections	30	60	90
Microfilariae* at necropsy	8.2 B	3.0 B	104.7 A

* Microfilariae per 20 μl of blood

** The letter postscripts are used to indicate statistical significance $p < 0.05$). Values with different letters are significantly different from those which share the same letter.

Table 2. Quantitation of lymphatic lesions in subcutaneously infected jirds in experiment 1

	Treatment	
	Subcutaneous inoculation	Intraperitoneal and subcutaneous inoculations
Mean lesion score	2.8**	1.9
Mean lesion score per worms in lymphatics	0.32*	0.11
Mean No. of thrombi per infected per spermatic cord lymphatics	5.1***	0.9
Mean No. of thrombi per worms in the spermatic cord lymphatics	1.18**	0.17
Mean weight of spermatic cord lymphatic drawings	0.173	0.196
Mean % increase in spermatic cord lymphatic size	266	314
Mean weight renal lymph node drawing	0.452	0.382
Mean % increase in renal lymph node size	176	134

* Significantly greater $p < 0.05$
** Significantly greater $p < 0.01$
*** Significantly greater $p < 0.001$

did not differ following SQ infection as determined by titers measured in sera taken at necropsy. The mean antibody titers of sera from the jirds receiving only the SQ inoculations reached levels (1:1024) similar to the other groups at necropsy.

The degree of lymphatic pathology which developed in animals receiving SQ infections measured by mean lesion scores, lesion scores per worms in lymphatics, intralymphatic thrombi per infected spermatic cord and thrombi per worm in the spermatic cord lymphatics was greater in animals without existing IP infections (Table 2). The increases in size of the spermatic cord lymphatics or renal lymph nodes were not significantly different between these groups. Lymphatic lesions were not observed in the jirds receiving only IP inoculations or in the uninfected controls.

Experiment 2

Significant differences were not noted in the parasitologic and lymphatic-pathologic observations made at necropsy on SQ infected jirds in groups receiving beads coupled with different substances (Table 3), and indicate that all groups had similar *B. pahangi* infections.

Comparisons of the mean pulmonary granuloma sizes that formed around beads coupled with different substances in jirds with different histories of exposure to *B. pahangi*-Ag is shown in Table 4. The jirds in the treatment groups differed significantly in their granulomatous responses to *B. pahangi*-Ag cou-

Table 3. Summary of parasitologic data and quantitation of lymphatic lesion severity of infected animals in experiment 2*

	Type of substance coupled to beads		
	B. pahangi antigen	BSA	DEA
Total worms recovered	19.0	14.0	16.0
No. of worms in lymphatics	17.8	14.0	14.2
No. of worms in nonlymphatic sites	1.2	0	1.8
No. of worms per spermatic cord lymphatic	6.8	4.4	5.8
Microfilariae** at necropsy	13.2	3.0	31.4
Lesion score	2.0	1.5	2.2
Lesion score per worms in lymphatics	0.12	0.13	0.20
No. of thrombi per infected spermatic cord lymphatic	0.9	1.1	2.6
Thrombi per worms in spermatic cord	0.18	0.25	0.60

* All numbers are mean values
** Microfilarial per 20 μl of blood

Table 4. Mean granuloma size* around beads coupled with *B. pahangi* antigen, BSA or Diethanolamine

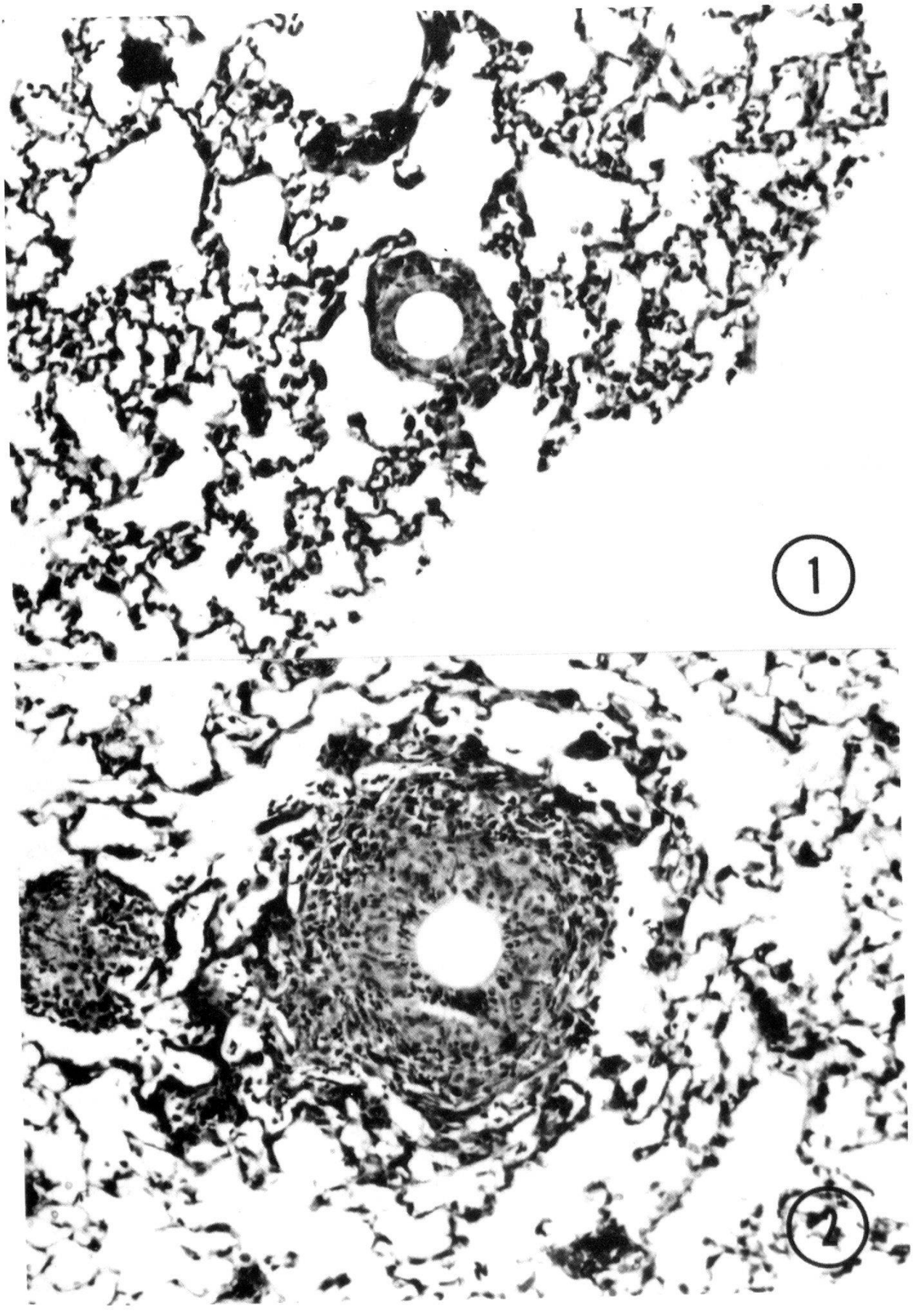
Treatment	Materials coupled to beads		
	B. pahangi antigen	BSA	Diethanolamine
B. pahangi infected	73 ± 20 C**	59 ± 10 D, E, F	60 ± 10 D, E
B. pahangi Ag in FCA	108 ± 22 A	57 ± 13 E, F	61 ± 13 D, E
None	83 ± 20 B	56 ± 9 F	63 ± 10 D

* Mean size in μm ± S. D.
** The letter postscripts are used to indicate statistical significance ($p < .05$). Values with different letters are significantly different and those which share letters are not.

pled beads but not to beads coupled with control substances, BSA or DEA. The most marked reaction to beads coupled with *B. pahangi*-Ag was observed in jirds hypersensitized with *B. pahangi*-Ag. Jirds infected with *B. pahangi* exhibited the smallest reaction to *B. pahangi*-Ag coupled beads. The reactions to beads coupled with *B. pahangi*-Ag was greater than that to both BSA and DEA coupled beads in jirds in all treatment groups. There was no significant differ-

Fig. 1. This section of lung from a *B. pahangi*-infected jird reveals a DEA coupled bead within a pulmonary vessel. There is endothelial swelling and a modest infiltration of the vessel wall and perivascular tissue with mononuclear leukocytes. 180×.

Fig. 2. This section of lung from a *B. pahangi*-Ag sensitized jird, reveals a bead coupled with *B. pahangi*-Ag within a pulmonary vessel. The vessel wall is obliterated by a moderate inflammatory cell infiltrate consisting of an inner zone of polymorphonuclear leukocytes and an outer zone of mononuclear leukocytes and epitheliod cells. 180×.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ence between the reactions to beads coupled with BSA and DEA either between treatment groups or within treatment groups.

The compositions of the small granulomas which formed around beads coupled with BSA and DEA in animals of all treatment groups were similar histologically. These were composed primarily of mononuclear cells (65 to 80%) with some neutrophils (12 to 30%) and fewer eosinophils (Fig. 1). The granulomas which formed around beads coupled with *B. pahangi*-Ag in *B. pahangi*-Ag sensitized jirds although larger were similar to these smaller granulomas in composition (Fig. 2). Granulomas which formed around beads coupled with *B. pahangi*-Ag in noninfected and infected animals were similar to each other but appeared to differ slightly in composition from the granulomas around beads coupled with BSA or DEA. These were composed of fewer monocytes (53 to 60%) and more eosinophils (25 to 37%) than neutrophils (10 to 16%).

Discussion

Results of both experiments, using different quantitative measurements, showed that pathologic tissue responses of jirds with existing *B. pahangi* infections to *B. pahangi* or *B. pahangi*-Ag were not as marked as those in jirds without previous infections. Although the methods used to measure reactivity to *B. pahangi* were different in these experiments there are indications that both lesions measured were similar. Previous histologic characterizations of the lymphatic lesions used to quantitate lymphatic pathology (Vincent et al., 1980; Klei et al., 1981) showed that these are primarily granulomatous in nature and similar in composition to the large granulomas which formed around the beads coupled to *B. pahangi*-Ag in the Ag sensitized jirds. Further, the *B. pahangi*-Ag sensitization protocol used in experiment 2, which produced the largest granulomas around beads coupled with *B. pahangi*-Ag was identical to the protocol used to hypersensitize jirds in previous experiments which resulted in increased lymphatic pathology in animals subsequently infected with *B. pahangi* (Klei et al., 1981). These observations indicated that either IP or SQ induced *B. pahangi* infections of 125 to 160 days duration in jirds induce mechanisms which suppress the granulomatous response to *B. pahangi*-Ag.

These hypo-responsive tissue reactions observed in the *B. pahangi*-jird model may correspond to the hypo-responsiveness described in human filariasis patients. In those studies peripheral blood lymphocytes recovered from microfilaremic patients commonly did not respond as strongly in vitro to filarial antigens as did those from amicrofilaremic patients (Ottesen et al., 1977; Piessen et al., 1980). In addition, Ottesen et al. (1977) observed an age difference in the occurrence of this unresponsive state and suggested that unresponsiveness developed as the infections became more chronic in nature. Mechanisms responsible for this hypo-responsiveness have been attributed to suppressor T-cells, humoral suppressor factors, and adherent mononuclear cells (Ottesen et

al., 1977; Piessens et al., 1980b). Mechanisms responsible for the decreased tissue reactions observed in our studies are unknown. However, earlier reports by Portaro et al. (1976) on in vitro spleen cell responses of *B. pahangi*-infected jirds to *B. pahangi* adult antigens indicated that an antigen specific unresponsive state developed 5 to 6 months following infection. Although our experiments were of a slightly shorter duration, similar mechanisms may be active.

Previous studies on recovery rates of fourth stage *B. pahangi* larvae from jirds with existing lymphatic populations of adult worms indicated that animals were less resistant to infection (Klei et al., 1980). Worm recovery data in this study differ and indicate that the development and location of larvae inoculated SQ into jirds with pre-existing IP infections was not significantly affected. The recovery rates of adult worms from jirds in both groups inoculated IP were similar but unexpectedly low when compared to previous reports using this technique (McCall et al., 1971). Microfilaremias also occurred in 3 jirds receiving only the intraperitoneal infections, and the levels of circulating microfilariae were significantly greater in jirds receiving both inoculations. The increased level of microfilariae in this group may be due to an additive effect of both infections, and/or the suppression of a mechanism which normally controls circulating microfilarial levels. Although unexplained, it is interesting to note that the lymphatic pathology in the SQ infected animals is most marked in the group with the lower microfilaremias and reduced numbers of patent infections. A similar association between amicrofilaremic states and increased lymphatic pathology was also noted in previous experiments using this model (Klei et al., 1981).

These previous studies linked amicrofilaremic states and increased lymphatic pathology with much higher antibody titers. The antibody titers observed in the hypo-responsive individuals of the present study were not as high as those reported previously in hypersensitized individuals but did not fall below levels observed in jirds with single SQ inoculations of 100 *B. pahangi* L_3. Similarly, jirds immunosuppressed with antithymocyte sera (ATS) had less severe lymphatic lesions following ATS treatment but had antibody titers like those of untreated jirds with more marked lymphatic lesions. These observations suggest that if the antibody and antigen complexes are responsible for the lymphatic lesions seen, these antibodies were not measured in the IHA assay, and/or that an antibody response is not solely responsible for the tissue reactions measured. Other studies have suggested that cellular immune phenomena may also be involved in the pathogenesis of lymphatic filarial lesions (Piessens et al., 1980b).

The pulmonary granulomas which formed around *B. pahangi* coated beads in unsensitized and uninfected individuals were significantly larger than those around BSA or DEA coated beads in similar jirds. This observation suggests that the *B. pahangi*-Ag used contains materials that nonspecifically induce

granulomatous inflammatory reactions. These factors may elicit these reactions directly or in combination with cells involved in the initial foreign body inflammatory reaction, causing these cells to release cytokines which potentiate the response. Other nematodes have been demonstrated to potentially enhance inflammatory reactions in these ways. An eosinophil chemotactic factor has been shown to be released in vitro from human neutrophils in the presence of *Nippostrongylus brasiliensis* larvae (Czarnetzki, 1978). Eosinophil chemotactic factors have also been isolated from extracts of *Anisakis* larvae, and demonstrated by in vitro and in vivo techniques (Tanaka and Torisu, 1978). The roles of nonspecific worm mediators of inflammation have not been addressed in lymphatic filariasis.

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