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Activation of Jird (*Meriones unguiculatus*) Macrophages by the Filarial Parasite *Brugia pahangi*

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Peritoneal macrophages from Mongolian jirds (*Meriones unguiculatus*) with either lymphatic or intraperitoneal infections of *Brugia pahangi* were studied to determine the effects of infection on macrophage function and morphology. Macrophages were collected at 40, 90, 140, and 200 days after inoculation of infective third-stage larvae and assayed for phagocytic and bactericidal activity by the acridine orange method and for morphological changes by light and electron microscopy. Significant increases in phagocytic and microbicidal activity ($P \leq 0.01$) were observed in peritoneal macrophages collected from jirds with intraperitoneal infections when compared with peritoneal macrophages from jirds with lymphatic infections and resident peritoneal macrophages from normal, noninfected jirds. Morphological changes in peritoneal macrophages from jirds with intraperitoneal infections were similar to those found in thioglycolate-elicited macrophage populations. Granuloma formation was also observed in the peritoneal cavities of intraperitoneally infected jirds. The peritoneal cavity may serve as a model to study cell-worm interactions in filarial nematode infections.

Lymphatic filariasis, caused by *Wuchereria bancrofti* and *Brugia malayi*, is a widespread parasitic disease affecting over 250 million people in the world (25). The acute stage of the disease is characterized primarily by lymphangitis caused by an inflammatory response to the worms or worm products. Recurrent episodes of lymphangitis lead to granuloma formation and eventual obstruction of the lymphatic vessels (19). These granulomas are composed primarily of macrophages, lymphocytes, and eosinophils.

The most widely used animal model for studying lymphatic filariasis is the Mongolian jird (*Meriones unguiculatus*) infected with *Brugia pahangi* (4, 5). Jirds subcutaneously (s.c.) inoculated with *B. pahangi* third-stage larvae develop a chronic, systemic lymphatic infection that resembles human filariasis (2). Jirds inoculated intraperitoneally (i.p.), however, develop infections which localize within the peritoneal cavity (4, 16, 18).

Klei et al. (15, 16) have reported that granuloma formation in the *B. pahangi*-jird model is immunologically mediated. Since macrophages are the primary cells involved in granulomas, it was of interest to determine whether *B. pahangi* infections activate these cells. Macrophage activation by chronic infections and its participation in granuloma formation have been well documented (7, 10, 13, 20). Activated macrophages therefore have a significant effect on the initiation, maintenance, and regulation of the granulomatous inflammatory response (3, 11, 23). Although activation of macrophages through immune induction involves specific antigen and immune lymphocytes, effector activity is nonspecific. Increased activation, such as increased microbicidal activity (23, 24) can be demonstrated against an antigenically different organism.

The purpose of the present study was to determine whether *B. pahangi* infections activate jird peritoneal macrophages, using phagocytosis and killing of *Staphylococcus aureus* and ultrastructural changes as indicators. s.c.- and i.p.-inoculated animals were studied at various times postinoculation to compare the effects of localized versus systemic infections on macrophages.

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MATERIALS AND METHODS

Animals and parasites. Ninety-six male and female inbred Mongolian jirds (*M. unguiculatus*; Tumblebrook Farms, West Brookfield, Mass.) less than 1 year of age were used. Lymphatic *B. pahangi* infections were initiated by s.c. inoculation of 100 infective larvae. i.p. infections were established by inoculation of 100 to 200 infective larvae. Infective larvae were obtained from infected mosquitoes (*Aedes aegypti*) provided by J. W. McCall, University of Georgia, Athens, through the U.S.-Japan Cooperative Medical Science Program of the National Institutes of Health. The infected mosquitoes were ground in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) containing penicillin, streptomycin, and HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), using methods previously described (1). Microfilariae were quantitated and adult worms were recovered at the time of necropsy by methods previously described (1, 16).

Elicited macrophages were obtained from normal, noninfected jirds by i.p. inoculation of 5 ml of sterile fluid thioglycolate (THIO; BBL Microbiology Systems, Cockeysville, Md.) 3 days before collection. These cells were used as a positive control for increased macrophage function. Resident peritoneal macrophages were obtained from noninfected, normal jirds and were considered nonactivated, control cells (CON). The s.c.-inoculated animals were sacrificed at 90, 140, and 200 days postinfection (DPI), and the i.p.-inoculated animals were sacrificed at 40, 90, 140, and 200 DPI. Cells from two THIO-treated and three CON jirds were collected and assayed each time an infected group of animals was killed.

Cell recovery. All animals were sacrificed with ether, and the cells were collected by peritoneal lavage. The peritoneal cavities were washed twice with 0.01 M phosphate-buffered saline, Ca^{2+} and Mg^{2+} free (pH 7.2), placed in sterile silicon-coated tubes, and kept at 4°C. The cells were centrifuged at 400 × *g* for 10 min at 4°C. The supernatants were removed, the cells were suspended in Gey buffer (pH 7.4), and cells from each treatment group were pooled.

Phagocytosis and killing function. Phagocytosis and killing

function were assayed by a modified method of Pantazis and Kniker, using acridine orange, a fluorochrome vital dye in which viable organisms fluoresce green, whereas dead ones fluoresce red (22). The peritoneal cells were diluted to a cell density of 10^6/ml in Gey buffer. The diluted cell suspension (1 ml) was added to 1 ml of an 18-h-old washed suspension of *S. aureus* in a ratio of 10 bacteria per cell. Pooled autologous serum was added to a concentration of 20%. The cell-staphylococcus mixture was incubated with shaking in a 37°C water bath for 30 min. The cells were centrifuged at 400 × *g* for 5 min at 4°C, the supernatant was removed, and the cells were suspended in 0.1 ml of Gey buffer and kept at 4°C until observed microscopically.

A drop of cell suspension and a drop of 0.14% acridine orange (Fisher Scientific Co., Fairlawn, N.J.) in Gey buffer were placed on a clean glass slide, and a cover slip was placed over the suspension. The cells were examined microscopically, using a Zeiss 63× oil immersion lens and a UV-light source equipped with a 510-nm filter. Two to three replicates of 50 cells each were counted for each treatment group. Mononuclear cells ingesting two or more fluorescing organisms were considered phagocytic cells. If these cells had two or more dead organisms within their cytoplasm, they were also considered as having killing activity.

Calculation of phagocytic and killing functions was performed as follows: percent phagocytosis = [(number of mononuclear cells with two or more organisms)/(total number of mononuclear cells counted)] × 100; percent killing = [(number of cells with two or more dead [red] organisms)/(total number of cells phagocytizing)] × 100.

Nomarski differential interference contrast microscopy was used to confirm that organisms were internalized within the cell and not on the surface of the cell.

Enumeration of peritoneal exudate cells. Smears of the cell suspensions were made and stained for nonspecific esterase activity by the method of Kaplow (14). Alpha-napthyl butyrate (Sigma Chemicals Co., St. Louis, Mo.) was used as the substrate, and 0.1% nuclear fast red (Sigma) was used as the counter stain. Differential counts were done with this stain, and total leukocyte counts were done by standard methods with a hemacytometer.

Electron microscopy. Macrophages were prepared for transmission electron microscopy by conventional techniques. Briefly, the cells were fixed in 1.25% glutaraldehyde–2.0% formaldehyde in 0.1 M sodium cacodylate buffer (NaCac) (pH 7.4) with 5.0% sucrose, postfixed in 1.0% osmium tetroxide in NaCac buffer, and then treated with 1.0% tannic acid in NaCac buffer. Cells were suspended in liquid 2.0% Sea Plaque agarose (FMC Corp., Rockland, Maine), and the solidified suspension was cut into 1-mm^3 blocks and embedded in epoxy resin. Thin sections were examined on a Zeiss EM 10 electron microscope.

Thick sections of each treatment group were cut approximately 0.5- to 1-μm thick and stained with methylene blue, and the cell types were counted according to their morphological characteristics by light microscopy. Two hundred cells from each group were counted.

Statistical analysis. Analysis of variance was performed on the raw data by a general linear models method. Mean treatment differences are reported as least-square differences.

RESULTS

Adult worm recoveries and location and peripheral blood microfilaria levels at the time of necropsy are summarized in Table 1. Total adult worm recoveries and percent recoveries were higher in the i.p.-inoculated jirds than in the s.c.-inoculated animals. Adult worms were primarily localized in the peritoneal cavity in i.p.-inoculated jirds, whereas in the s.c.-inoculated animals, worms were found in the heart and lymphatics. Microfilariae were present in the peripheral blood of i.p.-inoculated animals when adult worms were recovered from the lymphatics.

Total leukocyte counts and cell types from the peritoneal lavages are presented in Table 2. i.p.-inoculated animals at 40, 90, and 200 DPI had a significantly higher ($P \leq 0.01$) number of leukocytes than did the other treatment groups. The increase in leukocytes in the i.p.-inoculated jirds, however, was not linear with respect to DPI. The s.c.-inoculated jirds showed no difference in leukocyte numbers when compared with CON and THIO-inoculated animals. THIO-inoculated animals had significantly more peritoneal cells ($P \leq 0.01$) than did CON jirds. Formation of granulomas in the peritoneal cavity of i.p.-inoculated jirds appeared at 90 DPI and was present through 200 DPI. These granulomas were found both free and adhered to viscera and the peritoneum.

The cell types from the peritoneal cavities of the different treatment groups varied considerably. Granulocytes were present in large numbers in the 90- and 140-DPI i.p.-inoculated jirds and in the THIO-treated animals. These granulocytic cells were found in extremely small numbers in the peritoneal cavities of normal jirds and s.c.-inoculated animals. i.p.-inoculated jirds at all stages of infection had increased numbers of lymphocytes and nonspecific esterase-positive macrophages as compared with all other treatment groups. Nonspecific esterase activity appeared to be more intense in mononuclear cells that were small to medium in size, whereas the larger cells had more diffuse nonspecific esterase-staining characteristics.

A significant increase in the phagocytic function of peritoneal macrophages was seen in the THIO-, and the 140- and 200-DPI i.p.-inoculated jirds as compared with the other treatment groups (Table 3). Macrophage killing activity, however, was increased in the THIO-treated animals and all i.p.-inoculated jirds. s.c.-inoculated jird macrophage activity did not differ from normal jird macrophage function except at 90 DPI, when killing ability equaled that of the THIO-treated and i.p.-inoculated animals.

Four basic mononuclear cell types were observed in peritoneal exudates from all treatment groups. The cells were classified on the basis of morphology, using light microscopy, and correlated to ultrastructural observations. The four types of cells observed were: (i) small- to medium-sized, round, regularly shaped cells (Fig. 1A); (ii) large mononuclear cells with pseudopodium-like projections (Fig. 1B); (iii) large cells with pseudopodium-like projections and containing vacuoles (Fig. 1C); and (iv) large phagocytic cells (Fig. 1D). There were differences among the treatment groups in the predominant morphological cell type present, and the numbers found in the thick sections are summarized in Table 4. CON and s.c.-inolculated animals had more small round cells than did the THIO-treated or i.p.-inoculated animals. Large cells with pseudopodia were more evident in exudates from THIO-treated and i.p.-inoculated jirds. These were also the only groups that had phagocytic cells present in the cell preparations sampled for electron microscopy.

DISCUSSION

Peritoneal macrophages collected from jirds with peritoneal infections of *B. pahangi* were stimulated as determined by

TABLE 1. Summary of mean adult worm recoveries, adult worm locations, and microfiliariae levels at the time of necropsy

Treatment group	No.	Total recovery[a]	Adult worms located in[b]:			Microfilaria level[d]
			Heart	Peritoneum	Lymphatics[c]	
s.c. inoculation						
90 DPI	6	10.3 (10.3)	3.1 (30.7)	0 (0)	7.2 (69.5)	42.8
140 DPI	6	18.1 (18.1)	5.5 (30.4)	0.16 (0.09)	12.5 (69.1)	72.3
200 DPI	5	17.8 (17.8)	6.6 (37.1)	0 (0)	11.2 (62.9)	179.6
i.p. inoculation						
40 DPI	3	30.0 (18.0)	0 (0)	30 (100)	0 (0)	0.0
90 DPI	8	86.8 (43.4)	0 (0)	86.8 (100)	0 (0)	0.0
140 DPI	5	63.2 (63.2)	0 (0)	60 (94.9)	3.2 (5.1)	91.8
200 DPI	5	40.4 (40.4)	0 (0)	31.2 (77.2)	9.2 (22.7)	57.8

[a] Values are expressed as mean total number of worms recovered; values in parentheses are percentage of total worms inoculated initially.
[b] Values are expressed as mean total number of worms recovered from site; values in parentheses are percentage of total worms recovered.
[c] Lymphatics include spermatic cords, testes, and popliteal, renal, and lumbar lymph nodes.
[d] Values are expressed as mean number of microfilaria per 20 ml of peripheral blood.

increased microbicidal activity and morphological change. This stimulation does not appear to be a systemic activation because peritoneal macrophages from jirds inoculated s.c. that developed a systemic infection did not exhibit increased activity. These results are similar to the findings of James and Colley (12), who reported that i.p. infections of *Schistosoma mansoni* in mice increased nonspecific tumoricidal activity of peritoneal macrophages.

Phagocytic function of peritoneal macrophages from jirds inoculated i.p. increased at 140 DPI. This increase in phagocytosis coincided with an increase in the number of microfilariae in peritoneal exudates, suggesting that microfilarial factors may be involved in macrophage activation. Increases in phagocytosis were not observed in s.c.-inoculated jirds before or after the prepatent period, which also suggests that cell-worm contact is required for increased phagocytic function.

It appears that filarial disease has the same effect as other chronic infections on macrophage activation. Macrophage activation in i.p. infections was persistent throughout the 200-day experimental period. Elicited macrophages in peritoneal cavities are thought to be derived from blood monocytes which migrate to the peritoneal cavity and mature into macrophages (23). The reported half-life of peritoneal macrophages in mice is 20 to 40 days (23). If the half-life of jird peritoneal macrophages is similar, our observations suggest that *B. pahangi* stimulates continual recruitment of circulating monocytes to the peritoneal cavity, where they mature into macrophages. Birmingham and Jeska (6) also reported this prolonged enhancement of macrophage function with peritoneal macrophages from *Brucella abortus*-infected mice.

At 140 DPI, i.p.-inoculated animals showed a decrease in the number of peritoneal exudate cells. The mechanism causing the decline is unknown at this time. The 140-DPI i.p. infections are characterized by an apparent increase in the numbers of free microfilariae in the exudates, a decline in the numbers of adult worms, and an increased number of granulomas in the peritoneal cavity. It can be hypothesized that (i) a regulatory mechanism has been initiated controlling the influx of inflammatory cells to the peritoneal cavity; (ii) the decline in numbers of free cells can be attributed to the increased numbers of cells participating in granuloma formation, leaving fewer free cells in the lavage fluids; or (iii) the nature and/or the levels of the parasitic stimulus is changing. The increase in cell numbers at 200 DPI in i.p. infections could be in response to a change in the composition and/or concentration of inflammatory stimulus, possibly overcoming host regulatory mechanisms that may have previously been activated. Further experiments are in progress to elucidate the kinetics of i.p. granuloma formation and the nature of the inciting agent.

TABLE 2. Mean leukocyte counts and cell types from the peritoneal cavity of infected, CON, and THIO-treated jirds at the time of necropsy

Treatment group	No.	Mean no. of leukocytes (10^6 ± SEM)			
		Total	Granulocytes	Lymphocytes	Macrophages
Control					
Noninfected	33	5.6 ± 0.5	0.05 ± 0.03	1.90 ± 0.26	3.65 ± 0.39
THIO	25	20.8 ± 1.9	9.56 ± 1.38	1.25 ± 0.29	10.04 ± 0.94
s.c. inoculation					
90 DPI	6	11.8 ± 2.2	0.22 ± 0.15	5.23 ± 2.24	6.34 ± 0.13
140 DPI	6	9.6 ± 7.2	1.00 ± 0.08	2.02 ± 0.07	6.56 ± 1.13
200 DPI	5	11.5 ± 3.1	0.00 ± 0.00	2.23 ± 0.68	9.27 ± 2.50
i.p. inoculation					
40 DPI	3	57.3 ± 1.6	2.47 ± 1.70	16.99 ± 3.16	37.88 ± 6.58
90 DPI	8	158.5 ± 12.4	74.83 ± 23.07	15.32 ± 2.13	68.34 ± 8.52
140 DPI	5	29.0 ± 2.8	9.97 ± 2.99	7.05 ± 1.02	11.97 ± 0.86
200 DPI	5	52.0 ± 14.1	9.64 ± 5.92	12.24 ± 1.53	30.12 ± 9.78

TABLE 3. Phagocytosis and killing function of peritoneal macrophages from infected, CON, and THIO-treated jirds

Treatment group	%Phagocytosis[a]	% Killing[a]
Control		
Noninfected	20.16 ± 1.14^{A}	45.70 ± 5.51^{A}
THIO	50.77 ± 2.76^{B}	73.44 ± 4.05^{B}
s.c. inoculation		
90 DPI	16.24 ± 1.96^{A}	56.89 ± 10.12^{AB}
140 DPI	15.44 ± 2.18^{A}	37.84 ± 5.29^{A}
200 DPI	19.05 ± 3.10^{A}	39.41 ± 10.38^{A}
i.p. inoculation		
40 DPI	21.13 ± 2.51^{A}	67.27 ± 8.03^{B}
90 DPI	31.40 ± 2.01^{A}	90.07 ± 3.73^{B}
140 DPI	46.52 ± 3.57^{B}	83.06 ± 4.59^{B}
200 DPI	36.49 ± 2.49^{B}	72.19 ± 5.57^{B}

[a] Values are expressed as percent ± standard error of the mean. Means with the same letter are not significantly different ($P \leq 0.01$).

In our experiments, an increase in bactericidal activity preceded an increase in phagocytic function. Macrophages from i.p.-inoculated jirds had enhanced killing activity at all stages of the infection, although an increase in phagocytosis did not occur until 140 DPI. This suggests that phagocytic function and killing ability are independent of one another and could be induced by two independent mechanisms. It has been suggested by North (21) that macrophage activation is a heterogeneous activity. The varying degrees of activation seen could be a result of activation of different subpopulations of macrophages (8, 17), and/or a result of the stimulus affecting various macrophage functions to different degrees (24).

Morphologically, peritoneal cells from jirds with i.p. infections of *B. pahangi* resembled cells elicted by THIO from noninfected jirds and cells elicited by THIO, mineral oil, and peptone broth from other species (9). These observations support the functional studies indicating that macrophages from i.p.-inoculated jirds are activated and that a chronic

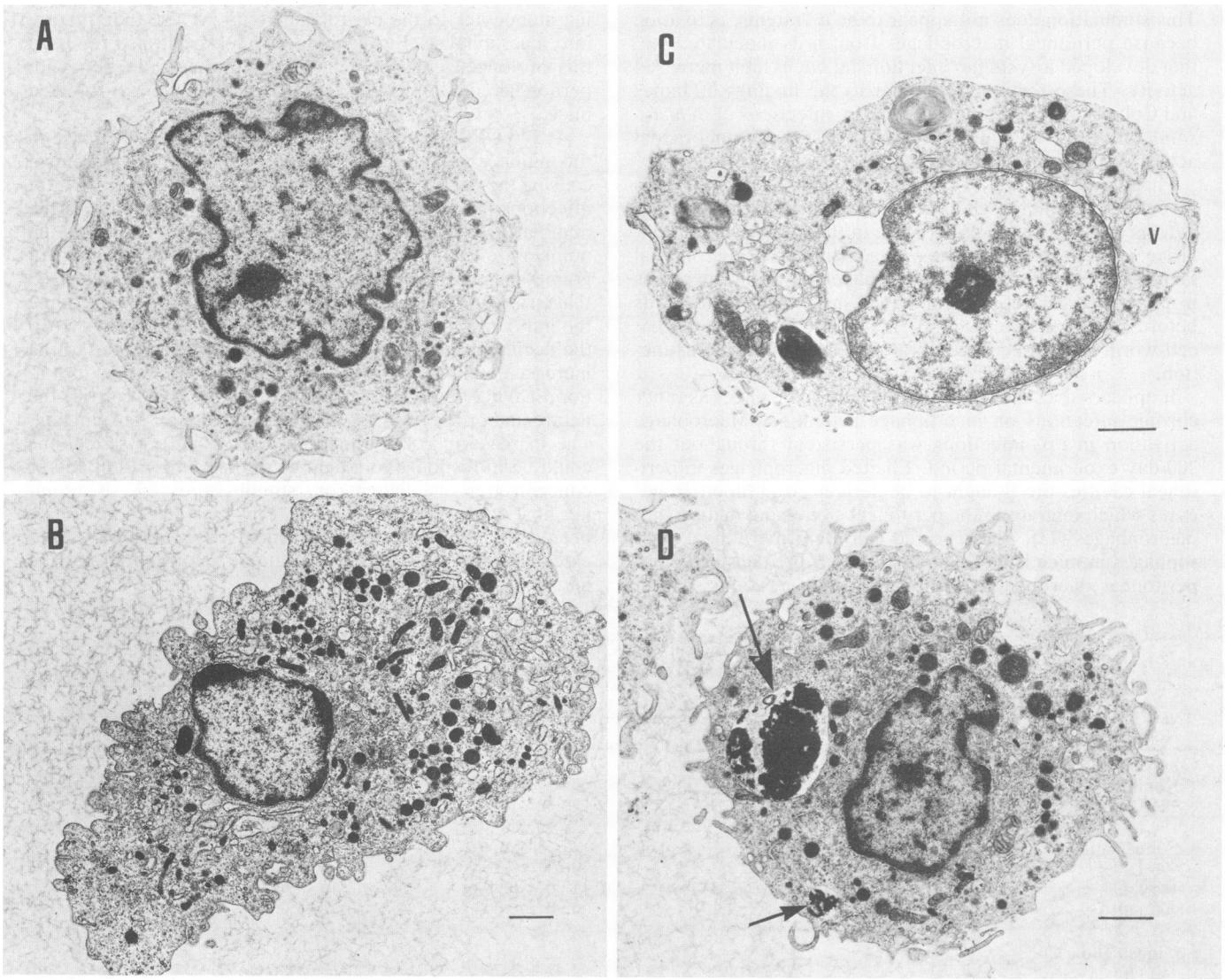

FIG. 1. Transmission electron micrographs of four morphological types of jird peritoneal macrophages. (A) Typical small, round mononuclear cell from a CON jird; (B) large mononulcear cell with many vacuoles and lysosomes from a 200-DPI jird with a peritoneal infection; (C) highly vacuolated (V) cell from a 3-DPI THIO-treated jird; (D) mononuclear phagocytic cell (see arrows) from a 200-DPI *B. pahangi* i.p. inoculated jird. Bar, 1 μm.

TABLE 4. Percentages of cell types from peritoneal cavities of infected, CON, and THIO-treated jirds based on morphology

Treatment group	Granulocytes	Mononuclear cells (%)[a]			
		SR	Pseud	Vac	Phag
Control					
Noninfected	0.0	58.5	41.5	2.5	0.0
THIO	20.0	5.0	44.0	30.5	0.5
s.c. inoculation					
90 DPI	3.0	52.0	31.5	13.5	0.0
140 DPI	0.0	58.5	38.0	3.5	0.0
200 DPI	4.0	34.0	59.0	3.0	0.0
i.p. inoculation					
40 DPI	17.0	42.0	38.0	2.5	0.5
90 DPI	24.0	5.0	40.5	24.0	6.5
140 DPI[b]	11.5	1.0	68.5	15.0	4.0
200 DPI[b]	13.5	1.0	67.0	15.0	3.5

[a] SR, Small to medium size, round (Fig. 1A); Pseud, large with pseudopodia (Fig. 1B); Vac, large with pseudopodia and vacuolated (Fig. 1C); Phag, large cell phagocytizing (Fig. 1D).
[b] Microfiliariae found in peritoneal exudate.

inflammatory response is occurring in the peritoneal cavity of these animals.

The presence of apparently parasite-activated macrophages and granulomas within the peritoneal cavity is significant. If macrophage activation and granuloma formation within the peritoneal cavity of i.p.-inoculated jirds is immune mediated, then the response may be similar to that described in lymphatic granuloma formation (15). The peritoneal model may thus be useful in studying cell-filarial interactions.

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