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IMMUNITY TO LARVAL *BRUGIA MALAYI* IN BALB/C MICE: PROTECTIVE IMMUNITY AND INHIBITION OF LARVAL DEVELOPMENT

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Abstract. The objective of this study was to analyze the immune response of mice to the larval stages of *Brugia malayi*. Male BALB/c mice were inoculated with 3 doses of irradiated third-stage larvae (L-3) of *B. malayi* and were subsequently challenged with L-3 implanted ip within diffusion chambers. After 3 weeks, larvae were recovered to determine their viability, length, and stage of development. A significant reduction in parasite survival was observed in immunized mice. Furthermore, larvae recovered from immunized mice were significantly shorter than larvae recovered from control mice. All larvae recovered from immunized mice were L-3, whereas 96% of larvae recovered from controls were fourth-stage larvae (L-4). Sera collected from control and immunized mice were tested for the presence of antibodies reactive with L-3 and L-4 antigens using an indirect fluorescent antibody assay employing frozen larval cross-sections as antigen. Sera recovered after challenge of control mice reacted with internal, but not surface, antigens of L-3 and L-4. Alternatively, sera from immunized mice reacted with both internal and external antigens of both L-3 and L-4.

Protective immunity to infection with *Brugia malayi* has been demonstrated in 2 rodent hosts, jirds and mice. Jirds were assessed for protective immunity by determining the number of adult worms recovered following challenge. Immunization consisted of a single subcutaneous inoculation of 15 Krad irradiated third-stage larvae (L-3). Eight weeks after immunization, the jirds were infected ip with L-3. Approximately 14% of L-3 injected into control jirds were recovered as adults, whereas only 1% were recovered from immunized jirds. This immunization regimen was thus successful at producing a 91% reduction in parasite survival.¹ Jirds have also been immunized against *B. malayi* using microfilarial antigens. Approximately 27% of L-3 injected into control jirds in this experiment were recovered as adults, whereas only 14% of challenge larvae were recovered from immunized jirds. This immune-mediated elimination of parasites in immunized jirds was only effective against female worms; equal numbers of male worms were recovered from control and immunized hosts.²

BALB/c mice were immunized by 3 sc injections of irradiated (20 Krad) *B. malayi* L-3. One week after the final immunization, mice were

challenged by ip injection of 100 normal *B. malayi* L-3. Two weeks after challenge, 15% of larvae injected into control mice were recovered live, whereas only approximately 1% were recovered from immunized mice.³ In another study, BALB/c mice were immunized by ip inoculations of non-irradiated worms. Challenge infections consisted of 50 L-3 injected ip. It was determined that about 20% of larvae injected into control mice survived for approximately 10 days, after which there was a rapid decline in the number of larvae recovered. In this study, resistance was evidenced following immunization by an accelerated elimination of challenge larval infections. Similar resistance to larval *B. malayi* was observed in mice immunized with live or dead L-3, or live fourth-stage larvae (L-4), male adult worms, female adult worms, or microfilariae.⁴

Studies on the development of larval filarids and on immunity to filarial larvae have been hindered by the difficulty of recovering larvae after inoculation into either natural or experimental definitive hosts. To overcome this obstacle, diffusion chambers have been utilized. This procedure places larvae in an in vivo environment yet still allows them to be completely and

efficiently recovered. Diffusion chambers have been used to maintain *B. pahangi*⁵ and *Onchocerca volvulus*⁶ in vivo, and to study immune responses to larvae of *Dipetalonema viteae*^{7, 8} and *Dirofilaria immitis*.^{9, 10}

The objective of the present study was to analyze the development of protective immunity to larval *B. malayi* confined within diffusion chambers. BALB/c mice were immunized using a previously described protocol³ and larval survival and development were assessed in control and immunized mice. Additionally, antibody responses to larval antigens were investigated to determine if a relationship existed between the patterns and intensity of antibody binding and immune protection.

MATERIALS AND METHODS

Aedes aegypti Liverpool (Blackeye strain) were infected with *B. malayi* by feeding on infected, anesthetized jirds originally obtained from the U.S.–Japan Cooperative Medical Science Program. Two weeks after infection, mosquitoes were surface sterilized in 95% ethanol followed by 1% benzalkonium chloride and were then placed on screens within culture medium filled funnels as previously described.⁸ L-3, collected from the funnels after 90 min, were placed in syringes, irradiated with 20 Krad gamma-radiation from a cesium-137 source and then injected subcutaneously into 10 week old male BALB/c mice (Jackson Laboratories, Bar Harbor, ME).

Immunization experiments were performed twice with differences between the 2 immunization regimens due to the availability of L-3. The immunization protocol used in Exp. 1 was: Day 0, 100 L-3; Day 14, 100 L-3; and Day 28, 30 L-3. The immunization protocol used in Experiment 2 was Day 0, 100 L-3; Day 14, 40 L-3; and Day 28, 40 L-3. In both experiments, challenge took place on Day 35 and necropsy on Day 56. Five mice were immunized in Exp. 1 and 3 in Exp. 2; 5 naive mice receiving challenge infections were used in each of the experiments. Challenge infections consisted of 20 L-3 implanted ip within diffusion chambers. Diffusion chambers were constructed from 14 mm lucite rings covered with 5 μ m pore size hydrophilic Durapore membranes (Millipore, Bedford, MA) as previously described.⁸

At the time of necropsy, mice were anesthe-

tized with methoxyflurane and then cervically dislocated. Diffusion chambers were recovered and the viable larvae counted. The recovered larvae were pooled by experimental group and then placed into glacial acetic acid followed by 70% ethanol containing 5% glycerin. The ethanol was allowed to evaporate, leaving the larvae in glycerin. Larvae were then measured by tracing their projected images with an opisometer. Larval stages were determined by the shape of the larval anterior end, as previously described.¹¹ A total of 58 larvae from control mice and 34 from immunized mice were measured and analyzed to determine the state of larval development.

Blood samples were collected from control and immunized mice prior to the implantation of the diffusion chambers and at the time of necropsy. Serum was separated from clotted blood by centrifugation and stored at -80°C . Frozen sections of L-3 and L-4 were prepared to test for the presence of antibodies in the mouse serum to larval antigens. L-3 were obtained as described above; L-4 were obtained from diffusion chambers that had been implanted in normal mice for 18 days. Larvae were embedded in O.C.T. compound (Miles Scientific, Naperville IL) and frozen on dry ice. Cryostat sections 10 μ m thick were collected on gelatin coated slides and stored at -40°C . Prior to labeling, sections were allowed to equilibrate to room temperature, and were then air dried for 15 min. Sections were permeabilized with 0.2% Triton-X in phosphate buffered saline (PBS) for 15 min at room temperature, briefly rinsed in PBS, and then incubated in neat goat serum for 1 hr at 37°C . Sera from individual control and experimental mice were diluted 1:10 in PBS containing 5% normal goat serum and then added to the sections of L-3 or L-4 for 1 hr at 37°C . The slides were washed 3 times in PBS for 5 min and incubated with a 1:300 dilution of FITC conjugated IgG fraction of goat anti-mouse IgM, IgG, and IgA (Cooper Biomedical, West Chester, PA) for 1 hr at 37°C . After 3 washes in PBS, slides were coverslipped with 90% glycerol in PBS with 0.1% p-phenylenediamine. To aid in the comparison of fluorescence between slides, photographs of sections were taken using equal exposure times, and the negatives subsequently compared for intensity of reaction. Photomicrographs used for the figures in this report used exposures that would best demonstrate the areas of fluorescence not the degree of fluorescence. Immunofluorescence

TABLE 1
Survival and growth of larval Brugia malayi in control and immunized mice

	Control	Immune
Exp. 1		
No. recovered larvae*	15	11
	12	11
	9	11
	7	5
	4	6
Mean % recovery	47 ± 21	44 ± 15
Mean larval length (mm)	2.87 ± 0.93 (33)†	1.79 ± 0.16 (25)
Exp. 2		
No. recovered larvae	20	13
	16	7
	16	5
	15	
	15	
Mean % recovery	82 ± 10	42 ± 21
Mean larval length (mm)	3.09 ± 0.77 (25)	2.12 ± 0.54 (9)

* Number of larvae recovered out of an initial inoculum of 20.

† Number of larvae measured.

was evaluated on a scale of (1)–(4) (weak diffuse fluorescence to brilliant fluorescence).

Statistical analyses of larval recovery and growth were accomplished using Student's *t*-test.

RESULTS

Larvae recovered 3 weeks after implantation in control and immunized mice were assessed for viability and development. Of larvae implanted in control mice in both experiments, 65% ± 24% were recovered live, as compared to 43% ± 16% of larvae from immunized mice (Table 1). This reduction of 34% in parasite survival was statistically significant ($P = 0.041$). Cellular adherence to the larval surface was a rare finding

for larvae recovered from both control and immunized mice. A significant difference in larval lengths was noted between larvae recovered from control mice and those recovered from immunized mice. L-3 measured directly after emergence from mosquitoes were 1.50 ± 0.16 mm long. Larvae recovered from control mice from both experiments were 2.97 ± 0.86 mm long, whereas larvae from immunized mice were only 1.88 ± 0.33 mm long ($P = 0.000$; Table 1). The percentage of larvae which developed from L-3 to L-4 was determined to be 96% for larvae recovered from control mice. In contrast, all larvae recovered from immunized mice were still L-3.

Antibodies binding to frozen sections of L-3 and L-4 were analyzed with serum collected from individual control and immune mice pre- and post-challenge. The results, summarized in Table 2, show that cryostat sections of L-3 and L-4 incubated in normal mouse sera displayed only a faint diffuse background fluorescence. After challenge, each control mouse produced a low level antibody response to internal antigens of both L-3 and L-4 (Fig. 1), but not to the surface of the larvae. No differences were noted in the antibody binding patterns to L-3 and L-4. Immunized mice produced moderate levels of antibody to both surface and internal antigens of L-3 and L-4 prior to challenge. After challenge, antibody binding to surface and internal antigens increased significantly (Fig. 2). Antibodies de-

TABLE 2
Relative antibody levels in the serum of control and immunized mice to the surface and internal antigens of Brugia malayi L-3 and L-4

	Control		Immune	
	Pre-challenge	Post-challenge	Pre-challenge	Post-challenge
L-3				
Surface	–	–	++	++++
Internal	–	+	++	++++
L-4				
Surface	–	–	++	++++
Internal	–	+	++	++++

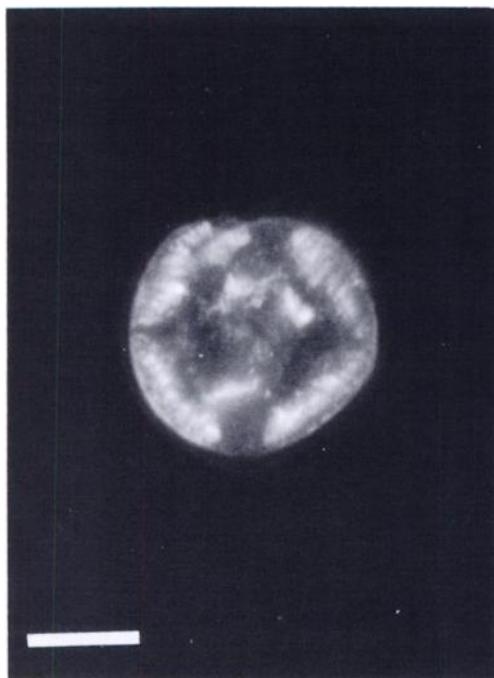


FIGURE 1. Indirect fluorescent antibody assay on cryostat section of *B. malayi* L-4 using sera collected from control mice following challenge. Similar results were obtained with L-3. Bar = 10 μ m.



FIGURE 2. Indirect fluorescent antibody assay on cryostat section of *B. malayi* L-3 using sera collected from immunized mice following challenge. Similar results were obtained with L-4. Bar = 10 μ m.

rived from all immune mice bound to L-3 and L-4 in an identical pattern.

DISCUSSION

B. malayi larvae survived in the peritoneal cavities of BALB/c mice for at least 3 weeks when implanted within diffusion chambers. Previous studies, in which larvae were inoculated directly into the peritoneal cavity of BALB/c mice, reported significantly fewer live larvae recovered after shorter infection durations.^{3,4} It is possible that the diffusion chambers provided a suitable environment for larval development in a normally insusceptible host, as has been suggested for the larvae of *D. immitis* in BALB/c mice.¹² Alternatively, larval recoveries from diffusion chambers may reflect a more accurate representation of the total number of larvae actually surviving within a host, in comparison to results obtained using other larval recovery methods. When *B. malayi* L-3 were implanted for 48 hr in diffusion chambers in the peritoneal cavities of *Mastomys natalensis*, rats, or jirds, recovery

rates of 35%, 24%, and 100% were obtained, respectively.¹³ Survival rates reported in the present study suggest that BALB/c mice, unlike *M. natalensis* and rats, are not resistant to infection with larval *B. malayi*.

A significant reduction in parasite survival was observed in challenge infections implanted in mice immunized with irradiated larvae. The reduction percentage found in the present study was much lower than that reported in previous studies for jirds¹ or mice³ immunized with irradiated larvae. It is difficult to make comparisons among these studies because of the different experimental protocols used. Furthermore, the recovery rate of worms from control animals in the previously mentioned studies was approximately 15%, whereas in the present study 65% of larvae inoculated into control mice were recovered. This difference in parasite recovery may effect the interpretation of relative immunization success observed for the different protocols. However, it may be concluded that *B. malayi*

larvae are susceptible to immune elimination in experimental rodent hosts.

Larvae implanted in immunized mice grew in length when compared to L-3 measured directly after emergence from the mosquito. The rate of growth in immunized mice was, however, significantly less than that seen for larvae implanted in control mice. It was further demonstrated that all of the L-3 implanted in immunized mice failed to develop into L-4. Growth retardation or arrested development of nematodes in immunized hosts has been reported for several species¹⁴ including *Ascaris suum*,¹⁵ *Heligmosomoides polygyrus*,¹⁶ and the filarial worms *Litomosoides carinii*,^{17, 18} *D. viteae*,^{7, 8} and *D. immitis*.¹⁰ It remains unknown whether *B. malayi* larvae in immunized mice have been subjected to permanently arrested development and will therefore remain as L-3, or if their development and growth is retarded but will eventually yield adult worms.

The relationship between immune-mediated retardation of larval development or growth and immune-mediated killing of larvae is unclear. There is evidence that the 2 phenomena are unrelated. *A. suum* larvae with growth retardation recovered from immune mice resumed their growth when transferred to non-immune mice.¹⁵ This finding suggests that the growth retardation of *A. suum* larvae does not have a permanent deleterious effect on the larvae. Jirds immunized against *D. viteae* with live or dead larvae eliminated significant portions of challenge infections. However, larval growth retardation was only seen in challenge infections recovered from jirds immunized with live larvae, but not in those immunized with dead larvae.⁷ Furthermore, if jirds were immunized with irradiated *D. viteae* larvae and the challenge infections were allowed to persist until adults developed, stunted growth was observed in the adult worms recovered from immunized jirds.¹⁹ These findings suggest that growth of *D. viteae* in jirds immunized with live larvae is diminished from L-3 to adult worms, but that development is unimpeded. Furthermore, killing and growth retardation of larvae appear to occur independently.

There is also evidence which suggests that immune-mediated retardation and killing of larvae are different aspects of a single phenomenon. Larval growth retardation was observed as a prelude to the elimination of *D. immitis* larvae from immunized dogs,¹⁰ and *D. viteae* larvae from immunized jirds.⁸ Differences in susceptibility to

immune reactions based on the larval phase of development were observed in both cases.^{8, 20} It was hypothesized that the protective immune response acts by retarding larval development at a stage with enhanced susceptibility to immune elimination. This would allow immune effector mechanisms sufficient time to interact with the susceptible stages.

Antibodies reactive with the internal and external antigens of L-3 and L-4 were present in mice after immunization and at significantly higher levels after challenge. Control mice developed antibodies to the internal components but not the surface antigens following challenge. A similar finding of elevated antibody responses to L-3 surface antigens following challenge has been reported for jirds immunized against *B. malayi*.¹ The fact that immune mice developed antibody responses to the larval surface whereas the controls did not suggests that surface antigens may be instrumental in inducing protective immunity. Additionally, the finding that both L-3 and L-4 antigens elicited antibody responses suggests that both of these antigenic sources must be investigated to determine their relative roles in the induction of protective immunity. This conclusion is supported by the report that either L-3 or L-4 were capable of immunizing mice to infection with larval *B. malayi*.⁴

The mechanism by which larval *B. malayi* are immunologically eliminated has been investigated in several studies. Spleen cells, but not serum, from immunized BALB/c mice were shown to be effective at transferring protective immunity to the larvae of *B. malayi*, thus implicating a cellular immune response.²¹ Death of *B. malayi* larvae implanted in diffusion chambers in *M. natalensis*, rats, and jirds was found to be correlated with the presence of cells within the diffusion chambers. The number of cells found within a diffusion chamber was negatively correlated by species to percent larval survival. If the diffusion chambers were constructed in a manner that would not permit cells to enter, larvae survived regardless of the host in which they were implanted.¹³ In the present study, the diffusion chambers used were covered with membranes that allowed cells unimpeded entry, yet finding cellular adherence to either live or dead larvae was a rare occurrence. Another mechanism for larval *B. malayi* destruction has been described based on results obtained from immunized jirds. Dead or dying larvae from chal-

lenge infections were observed within nodules in immunized but not control jirds. Eosinophils were found in close association with the moribund larvae. Based on the reported presence of antibodies reactive with the L-3 larval surface, it was suggested that challenge larvae were being eliminated by an antibody dependent eosinophil-mediated mechanism.²²

The mechanism by which larval development is retarded is unknown. It has been demonstrated that retarded development and stunting of *H. polygyrus* larvae is mediated by antibody directed at excretory/secretory products and not to surface antigens.¹⁶ A similar mechanism may exist for *B. malayi*.

The present study demonstrates that BALB/c mice can be immunized against infection with *B. malayi*. The immunity is characterized by a reduction in parasite survival, growth, and development. Although an association between larval killing and antibody to larval surface antigens was observed, the mechanisms of protective immunity and the antigens capable of inducing protective immunity remain to be determined.

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