Cellular and Humoral Immune Responses of Jirds Resistant to *Dipetalonema viteae* Infection

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Jirds with prepatent *Dipetalonema viteae* infections develop an acquired immunity to challenge infections. The objective of the present study was to observe parasite-specific and nonspecific cellular and humoral immune responses in immune jirds. Splenic hyperplasia was observed in infected jirds during the first 5 weeks of infection. Antigen-reactive spleen cells were observed in the lymphocyte transformation assay at 3 weeks postinfection. A depressed response to concanavalin A (ConA) was seen at 1 week postinfection through week 5. Mitomycin C-treated cells from infected jirds were capable of suppressing the response of normal cells to ConA. Sephadex G-10-nonadherent spleen cells from infected jirds showed elevated responses to *D. viteae* antigen at 1, 3, and 5 weeks and elevated responses to ConA at 3 and 5 weeks. Filaria-specific antibodies were seen at 1 week postinfection, and titers rose through week 5. Plaque-forming cell production to sheep erythrocytes was not depressed in infected jirds. It was concluded that jirds react immunologically with both cellular and humoral responses during the prepatent period of *D. viteae* infection. A concurrent immune depression was seen. Its effect on resistance and tolerance remains to be determined.

Filarial worm life cycles within the mammalian host consist of several different stages, all of which are capable of producing pathological changes (5, 23, 27, 33). For this reason, efforts have been made to protect hosts from larval filarial worms, as this prevents the development of all subsequent stages and pathological changes. Anthelmintics such as diethylcarbamazine (7-9, 16) and ivermectin (3) have been shown to be efficacious against larval stages of certain filariids. These agents are not widely available or for various reasons may lack utility. Another approach to preventing infection with filarial worms is immunization. Various protocols have been followed to immunize animals successfully against challenge filarial worm infections (4, 6, 14, 22). While there has been success in the laboratory, a practical method has not been developed for use under endemic conditions.

Dipetalonema viteae is a natural parasite of some gerbil species and can experimentally infect the Mongolian gerbil (jird) *Meriones unguiculatus* (39). Jirds infected with *D. viteae* have been shown to be resistant to challenge infections (1, 36). This immunity is present during the prepatent period of primary infections. The mechanism of larval destruction in vivo has not been elucidated, though there is evidence from in vitro experiments that larval *D. viteae* can be killed by serum (37) and cells (1, 13).

To better understand the development, as well as the mechanisms, of immunity to filarial infections, jirds with prepatent *D. viteae* infections were observed in the present investigation to determine the kinetics of filaria-specific humoral and cellular responses. Additionally, experiments were conducted to determine what effect the infection might have on the ability of an animal to respond to a mitogen or heterologous antigen.

MATERIALS AND METHODS

Host and parasite. All animals in these studies were inbred male jirds (*M. unguiculatus*) aged 3 to 6 months obtained from Tumblebrook Farm (West Brookfield, Maine) or bred from a mated pair originally obtained from Tumblebrook Farm. *D. viteae* was maintained in the soft tick *Ornithodoros tartakovskyi*. Third-stage infective larvae (L₃) were obtained from ticks 30 days after feeding on microfilaremic jirds which had been anesthetized with sodium pentobarbital, 0.1 ml/10 g of body weight.

Splenic indices. Splenic indices were calculated [(weight of spleen/weight of animal) × 1,000] for jirds receiving the following treatments: (i) subcutaneous injection of 30 L₃ in 0.3 ml of phosphate-buffered saline (PBS); (ii) subcutaneous injection of 0.3 ml of PBS in which normal ticks had been dissected; (iii) subcutaneous injection of 0.3 ml of PBS in which infected ticks had been dissected; (iv) being fed upon by three infected ticks; and (v) being fed upon by three normal ticks. Jirds were euthanized by ether inhalation 1 to 5 weeks after receiving one of the treatments. The spleens were excised, weighed, and fixed and sectioned by standard histological techniques. Four animals were used in each experimental group.

Lymphocyte transformation assays. Adult male *D. viteae* recovered from hamsters were ground in a tissue homogenizer, and the homogenate was extracted overnight in PBS at 4°C. The homogenate was centrifuged, diafiltered at a 10-kilodalton exclusion limit (Amicon Corp., Lexington, Mass.), filter sterilized, and stored at -70°C at a protein concentration of 1 mg/ml. This antigen was used in the lymphocyte transformation assays.

Normal jirds and jirds infected with 30 *D. viteae* L₃ for 1, 3, or 5 weeks were euthanized, and their spleens were excised, minced, and passed through a steel mesh screen. Cells were collected in cold RPMI 1640 with 5% fetal calf serum and centrifuged, and erythrocytes were lysed with Tris-buffered ammonium chloride. After three washes in RPMI 1640, the cells were counted, and their viability was assessed by dye exclusion. Cells (4 × 10⁵) in 0.2 ml of RPMI

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FIG. 1. Splenic indices for jirds that were inoculated with 30 L₃ or PBS in which normal or infected ticks had been dissected or that were fed upon by normal or infected ticks. Spleens were recovered 1, 3, or 5 weeks after treatment. a and b, $P < 0.01$; b and c, Not significant; d and e, Not significant.

1640 with 10% fetal calf serum, 200 μ g of kanamycin per ml, and 500 μ g of neomycin per ml were added to each well of a 96-well round-bottom plate. A sample of 1 μ g of concanavalin A (ConA) (Calbiochem-Behring, La Jolla, Calif.) or 5 μ g of *D. viteae* antigen was added in 20 μ l of culture medium to triplicate cultures. 2-Mercaptoethanol (Eastman Kodak Co., Rochester, N.Y.) was added in 20 μ l of culture medium to all wells to yield a 5×10^{-5} M solution. After 48 h, 20 μ l of tritiated thymidine (10 μ Ci/ml) (New England Nuclear Corp., Boston, Mass.) was added to each well. After 24 h, cells were harvested (MASH; Flow Laboratories, Inc., McLean, Va.), and incorporation of tritiated thymidine was determined with a liquid scintillation spectrophotometer.

Cells were treated with mitomycin C (Sigma Chemical Co., St. Louis, Mo.) or separated on a Sephadex G-10 (Pharmacia, Inc., Piscataway, N.J.) column to test for the presence of suppressor cells. Spleen cells from normal jirds and jirds infected with 30 *D. viteae* L₃ for 2 weeks were treated with mitomycin C (40 μ g/ml at 37°C for 30 min). Pooled normal spleen cells (2×10^5) were added to either 2×10^5 mitomycin C-treated pooled normal cells or 2×10^5 mitomycin C-treated cells from three infected jirds. Lymphocyte transformation assays without 2-mercaptoethanol were performed on these cells.

Sephadex G-10 was hydrated, washed, and autoclaved before use. Gel (8 ml) was added to a column, washed with PBS and then RPMI 1640 with 10% fetal calf serum, and equilibrated at 37°C before the addition of cells. Cells (5×10^7) in 2 ml of prewarmed medium were added to each column; after the cells entered the column, 20 ml of additional medium was added. The recovered nonadherent cells from three to six animals were then used in blast-transformation assays.

Determination of filaria-specific antibody by ELISA. The methods used for preparation of antigen and for performing the enzyme-linked immunosorbent assay (ELISA) for filaria-specific antibody detection were based on those of Grieve et al. (11). Adult male and female *Dirofilaria immitis* worms were used as the antigen source. A peroxidase-conjugated

immunoglobulin G fraction of goat anti-hamster immunoglobulin G (heavy and light chains) (Cappel Laboratories, Cochranville, Pa.) was used as the conjugated second antibody in an indirect ELISA. Antibody levels were determined by spectrophotometric readings of serially diluted sera. A minimum of nine animals were used in each experimental group.

Plaque-forming response to sheep erythrocytes. Normal jirds and jirds infected for either 2 or 3 weeks with 30 L₃ were inoculated intraperitoneally with 2×10^8 sheep erythrocytes. After 1 week, spleens were removed, a single cell suspension was prepared, and the cells were used in a hemolytic plaque assay, as described by Mishell et al. (21). Four animals were used in each experimental group.

Statistical analysis. All data were evaluated for statistical significance by using Student's *t* test.

RESULTS

Spleens from jirds infected with 30 L₃ were markedly enlarged during the first 5 weeks of infection. Splenic indices were maximal at 1 week postinfection, and were still double that of normal at 5 weeks postinfection. Inoculation of PBS in which normal or infected ticks had been dissected produced elevated splenic indices 1 week postinoculation. These indices reverted to normal by 3 weeks postinoculation. Feeding of infected ticks on jirds stimulated splenic hyperplasia equivalent to that seen when larvae were injected. Being fed upon by normal ticks did not induce an increase in spleen size (Fig. 1).

The predominant cause for the increase in spleen weight in jirds infected for 1 week with 30 L₃ was an expansion of the red pulp. Macrophages present in the red pulp increased in size and number. There was also an enlargement of the white pulp due to an increase in the size and number of lymphoid cells. These lymphoid cells were homogeneous throughout the white pulp, and an increase in mitotic activity in these cells was observed. Two weeks after infection with 30 L₃, the red pulp remained as described above. The expansion of the white pulp, however, was the predominant cause of the

FIG. 2. Lymphocyte transformation responses of spleen cells and Sephadex G-10-nonadherent spleen cells to *D. viteae* antigen. Cells taken from normal jirds and jirds infected with 30 L_3 for 1, 3, or 5 weeks. *, Not significant; **, $P < 0.01$.

splenomegaly. Large blastlike cells were intermixed with the normal lymphocytes in the white pulp. Lymphocytes expanded in a perivascular fashion into the red pulp. By 3 weeks postinfection, the red pulp returned to normal size. Macrophage numbers and sizes reverted to previous levels, and the sinusoids once again were obvious. The perivascular extensions from the white pulp, however, continued to expand. The lymphoid cells in the white pulp remained homogeneous, and large blastlike cells were noted. No histological differences were observed between spleens from jirds 3 weeks after inoculation of 30 L_3 , or between spleens from jirds receiving bites from infected ticks.

A significant lymphocyte transformation response to *D. viteae* antigen was observed for spleen cells recovered from jirds with 3- and 5-week infections but not for cells from animals with 1-week infections (Fig. 2). A depressed re-

sponse to ConA was evident in spleen cells from animals with 1-, 3-, and 5-week infections (Fig. 3).

Mitomycin C-treated spleen cells from animals with 2-week infections were capable of suppressing the response of normal spleen cells to ConA. No immunodepression was observed when mitomycin C-treated spleen cells from normal animals were added to untreated cells (Fig. 4).

Approximately 48% of normal cells and 28% of cells from immunized hosts were recovered after passage through Sephadex G-10. Sephadex G-10-nonadherent spleen cells from infected animals showed enhanced responses to *D. viteae* antigen compared with their source populations (Fig. 2). Removal of adherent cells also greatly increased the response to ConA by cells recovered from jirds with 3- and 5-week infections but not for cells from jirds with 1-week infections (Fig. 3).

FIG. 3. Lymphocyte transformation responses of spleen cells and Sephadex G-10-nonadherent spleen cells to ConA. Cells taken from normal jirds and jirds infected with 30 L_3 of *D. viteae* for 1, 3, and 5 weeks. *, Not significant; **, $P < 0.01$.

FIG. 4. Lymphocyte transformation responses of normal jird spleen cells to ConA after the addition of mitomycin C-treated spleen cells recovered from either normal jirds or jirds with 2-week 30 L_3 infections.

A significant antibody response was observed at 1 week postinfection with titers increasing through the subsequent 4 weeks (Fig. 5). No significant differences were noted in the numbers of plaque-forming cells to sheep erythrocytes found per spleen in normal animals ($31,952 \pm 2,075$) compared with spleens from animals infected for 2 or 3 weeks ($43,720 \pm 16,550$).

DISCUSSION

Splenomegaly has been observed in hamsters infected with *D. viteae* (40), cotton rats and multimammate rats infected with *Litomosoides carinii* (30, 43), and jirds infected with *Brugia pahangi* (15, 38), *Brugia malayi*, and *Brugia*

patei (38). Each of these reports describes the requirement of a microfilaremia for the development of splenic hyperplasia. In the present study, increased splenic indices were observed in infected jirds during the prepatent period, thus suggesting a different etiology for the splenomegaly.

There appeared to be two stimuli responsible for the splenic hyperplasia. The first, which had a short-term effect, was noted after inoculation with both infected and uninfected tick homogenates. It has been hypothesized that *O. tartakowskii* has very potent antigens, such as tick salivary enzymes, which may play an important role in the rapid basophil recruitment to the tick bite (20). Similar antigens may play a role in the rapid but short-lived development of splenomegaly in jirds injected with tick material.

The second stimulus for the splenomegaly was the presence of L_3 regardless of whether the larvae were injected by syringe or deposited during feeding by infected ticks. The finding that infected-tick bites caused splenomegaly, whereas uninfected-tick bites did not, supports the conclusion that the splenic hyperplasia is not a laboratory artifact, and that it is larva induced.

D. viteae antigen-reactive cells, as demonstrated by the lymphocyte transformation assay, were present in the spleens of jirds with 3- and 5-week infections but not with 1-week infections. The time at which antigen-reactive cells were first detected coincided with the time at which the splenic white pulp expansion was noted. The finding of antigen-reactive cells at this time is consistent with observations of hamsters infected with *D. viteae* (31) and Lewis rats infected with *B. pahangi* (42).

A depressed response to ConA was first observed at 1 week postinfection. Immunodepression has been previously observed in many other filarial infections, as reviewed by Haque et al. (12), though not during the prepatent phase of the life cycle.

There are two lines of evidence from the present study that suggest that nonspecific depression seen in jirds with prepatent *D. viteae* infections was due to suppressor cells. The first is that mitomycin C-treated cells from infected animals were capable of suppressing the response of normal cells to ConA. Secondly, the suppression seen in whole spleen cell populations could be eliminated by removing cells adherent to Sephadex G-10. Removal of G-10-adherent

FIG. 5. Antibody titers against adult *D. immitis* antigen in serum from normal jirds and jirds infected with 30 L_3 of *D. viteae* for 1, 2, 3, and 5 weeks. *, $P < 0.02$; **, $P < 0.01$.

cells enhanced the response to antigen by cells from 1-, 3-, or 5-week infections and to mitogen by cells from 3- and 5-week infections.

Adherent suppressor cells have been associated with two other filarial infections. Lammie and Katz (17–19) have demonstrated specific and nonspecific adherent suppressor cells in *B. pahangi*-infected jirds; these cells appear after the onset of microfilaremia. Piessens et al. (28) reported the presence of adherent suppressor cells in *B. malayi*-infected patients. These cells are also present only after patency and are specific for filarial antigen.

The presence of an immunodepression appears to have little effect on the generation of humoral responses to *D. viteae* in the inbred jird. Significant antibody titers were observed at times similar to those previously reported for *D. viteae* infections of the outbred jird (26), hamster (40, 41), white rat (32), and multimammate rat (35). Additionally, these times coincide with times reported for the appearance of significant antibody titers for *B. malayi* in the jird (34), *B. pahangi* in the cat (2, 29, 31), and *D. immitis* in the dog (10, 25). A functional humoral response in the presence of depressed cellular responses has been reported by Ottesen et al. (24). They observed patients with chronic bancroftian filariasis who, while showing a state of specific cellular unresponsiveness, show no differences in total or filaria-specific antibody levels as compared with nonsuppressed patients.

Humoral responses to heterologous antigens also appeared unaffected by the immune depression. No differences in plaque-forming cell development was seen between the control and infected animals. The finding of an unaltered plaque-forming cell response to a heterologous antigen in the presence of immune depression has been reported by Lammie and Katz (18) for jirds infected with *B. pahangi*.

In conclusion, jirds develop cellular and humoral responses to infection with *D. viteae* early in the prepatent period. This finding is consistent with immune resistance present at this time, as well as the mechanisms suggested for the resistance. Concurrent with the specific immune responsiveness, a nonspecific cellular immune depression was observed in jirds with apparently normal humoral responses to filarial and nonfilarial antigens. The role that the immune depression plays in relation to parasite survival or destruction thus remains unclear.

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