EXPERIMENTAL PARASITOLOGY 60, 263–269 (1985)

Ancylostoma ceylanicum: Immunization with Soluble Worm Extract and Responses to Challenge Infection of Dogs

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(Accepted for publication 11 February 1985)

CARROLL, S. M., AND GROVE, D. I. 1985. *Ancylostoma ceylanicum:* Immunization with soluble worm extract and responses to challenge infection of dogs. *Experimental Parasitology* **60**, 263–269. When dogs were immunized with soluble extract of adult *Ancylostoma ceylanicum* antigen, they were partially resistant to challenge infection in this model of human hookworm infection. Two immunizing doses, each of 1 mg protein suspended in Freund's complete adjuvant, were administered to one group of animals 1 and 3 weeks prior to infection with 5000 larvae. When compared with control dogs given the same infective dose, fecal egg excretion and intestinal adult worm burden in the immunized animals were reduced by 59 and 74%, respectively. Infection had no significant effect on hemoglobin concentrations, mean red cell volumes, total white cell counts, platelet levels, or spontaneous and phytohemagglutinin-induced lymphocyte transformations in both control and immunized animals. Both groups developed an eosinophilia, and lymphocytes from the immunized dogs responded transiently to stimulation with both larval and adult worm antigens. Specific IgM antibodies were transitory in both groups of dogs following infection. IgG antibodies developed significantly 2 weeks after infection in the immunized group; however, they did not appear until 4 weeks after infection in the control group. Both groups developed IgA antibodies 1 week after infection. They were maintained in the control dogs, in contrast to the levels in immunized animals which subsided rapidly 4 weeks after infection. Therefore, when animals are injected with soluble adult worm antigen prior to infection, specific protective immunity is acquired.

INDEX DESCRIPTORS: *Ancylostoma ceylanicum;* Nematode, parasitic; Hookworm, human; Dogs; Immunization; Challenge infection.

INTRODUCTION

Hookworm infection is a major cause of ill-health in humans, particularly in developing countries. Various strategies have been proposed for the control of intestinal helminthiasis, including mass chemotherapy and improved environmental sanitation and health education. Unfortunately, although effective anthelmintics are available, mass administration frequently fails to control such infections as reinfection is common (Docherty 1926; Cort *et al.* 1929; Bhaibulaya *et al.* 1977). Similarly, it is unlikely that the standards of living in many of the affected countries will improve in the foreseeable future.

An alternative approach is the development of vaccines which stimulate persistent immunity with a consequent reduction in worm burden and elimination of disease. We have recently reported a canine model of human ancylostomiasis using *Ancylostoma ceylanicum* (Carroll and Grove 1984) and have used this system to investigate relationships between this parasite and its host. Considerable resistance to reinfection was observed following termination of chronic infections with anthelmintics (Carroll and Grove a, in press). Similarly, a current infection with small numbers of worms

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0014-4894/85 $3.00

conferred significant resistance to superinfection with large numbers of larvae (Carroll and Grove b, in press). We now report the use of this model to determine whether it is possible to induce immunity to infection by injection of soluble *A. ceylanicum* antigens prepared from adult worms. In addition, we have measured associated hematological and immunological responses to infection.

Materials and Methods

Ancylostoma ceylanicum was obtained originally from Malaysia. The acquisition of the parasite, maintenance of the life cycle, and methods of infection have been described in detail elsewhere (Carroll *et al.* 1983). This strain of *A. ceylanicum* has subsequently produced patent infections in humans (Carroll and Grove c, in press).

Soluble antigen was prepared from adult *A. ceylanicum* worms recovered at autopsy from the intestine of an infected dog as has been described for *Strongyloides ratti* (Carroll *et al.* 1981). Briefly, bacterial contaminants were removed by repeatedly washing the adult worms trapped on 8.0-μm filters (Millipore Corp., Bedford, MA, USA) with sterile 0.9% saline. Worms were ground at 4 C in Pyrex tissue grinders, incubated overnight at the same temperature, and then centrifuged for 60 min at 30,000g. The supernatant fluid was filter-sterilized by passage through a Millex-GV 0.22-μm filter (Millipore). The protein concentration was determined by the method of Hartree (1972). The antigen was then stored at −20 C.

The techniques for measuring fecal egg excretion, intestinal adult worm burdens, hematological parameters, and lymphocyte transformations have been described earlier (Carrol and Grove 1984).

Serum antihookworm antibody levels were measured by an enzyme-linked immunosorbent assay similar to that described elsewhere (Carroll *et al.* 1981), with certain modifications. Antigens were prepared from *A. ceylanicum* as described above. Larval extract antigen was used for IgM antibody detection, and partially purified antigens from adult worms, D_1 and D_2, were used for IgG and IgA antihookworm antibody detection, respectively; these antigens are fractions of adult worm antigen which were separated by Sephadex-G200 column chromatography. Preliminary tests had shown these fractions were the most discriminatory in measuring IgG and IgA antibody activity. Microtiter trays (Linbro; Flow Laboratories, McLean, VA, USA) were coated with 10 μg/ml of antigen prepared in 0.1 *M* carbonate–bicarbonate buffer, pH 9.6, by incubation of trays for 60 min at 37 C and then overnight at 4 C. All test sera were diluted 1:50 in wash buffer (0.1 *M* phosphate buffer with 0.5% Tween 20) containing 10% fetal bovine serum; plates were washed using an automated plate washer (Multiwash; SkatronAS, Lier, Norway); and all incubation steps were for 60 min at 37 C. IgM and IgG antibodies were measured using optimally diluted goat antidog IgM and IgG antisera, respectively (Cappel Laboratories, Cochraneville, PA, USA) followed by alkaline phosphatase-labeled swine antigoat IgG antibody (Tago, Burlingame, CA, USA); whereas specific IgA antibody was measured with rabbit antidog IgA antibody (Pel-Freez Biologicals, Rogers, AR, USA) followed by alkaline phosphatase-labeled goat antirabbit IgG antibody (Tago). Color development using *p*-nitrophenyl phosphate (1 mg/ml) was stopped after 60 min with 1 N NaOH, and the optical densities were read at 405 nm using an automated plate reader (Titertek Multiscan; Flow).

Four male mongrel dogs between 6 and 9 months in age and weighing between 6 and 13 kg were immunized by multiple subcutaneous injections in the inguinal region with adult worm antigen in Freund's complete adjuvant. Two immunizing doses, each of 1 mg protein (total, 2 mg), were administered on Weeks 1 and 3 of the experiment. On Week 4, these dogs together with four control animals were infected with 5000 infective larvae percutaneously. One animal of the control group became ill during the experiment and was removed from the study. Feces were collected weekly, and blood samples were obtained at 10 AM on the same day. Six weeks after infection, animals were killed, the intestines were opened longitudinally, and adult worms were removed, sexed, and counted.

All results are expressed as means ± standard deviation. All tests of significance were performed using the two-tailed Student's *t* test.

Results

Eggs were first seen in the stools 3 weeks after infection with *Ancylostoma ceylanicum*. Four, five, and six weeks after infection, the reduction in fecal egg output in immunized animals compared with control dogs was 58, 53, and 67%, respectively (mean 59%). These reductions were statistically significant (Fig. 1).

Dogs were examined at autopsy 6 weeks after infection. There was a 74% reduction in adult hookworm numbers recovered from immunized dogs, the mean values being 1400 ± 590 and 358 ± 55 for control and immunized dogs, respectively (Fig. 2); these values represent recoveries of 28 and

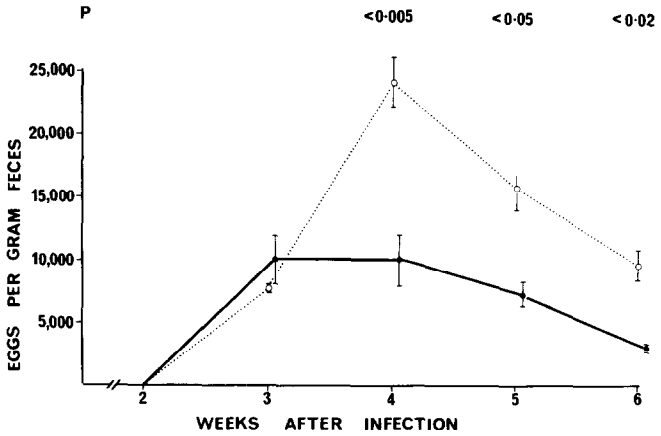

FIG. 1. Fecal *Ancylostoma ceylanicum* egg excretion of immunized (●) and control (○) dogs at weekly intervals after infection. Three dogs were in the immunized group and four dogs in the control group. Results are expressed as the means ± SEM.

7% of the infecting dose, respectively. The difference in adult worm burden was statistically significant ($P < 0.05$). The mean percentages of female worms were 58 ± 2 and 65 ± 4 in the control and immunized animals, respectively; this difference was not statistically significant.

There was no significant difference in the hemoglobin concentrations between the control and immunized dogs prior to infection, the values being 13.4 ± 1.7 and 14.7 ± 2.0 g/dl, respectively. Following infection, hemoglobin concentrations fell in both groups (minimum value, 10.4 ± 1.1 g/dl), but there were no significant differences within or between the 2 groups.

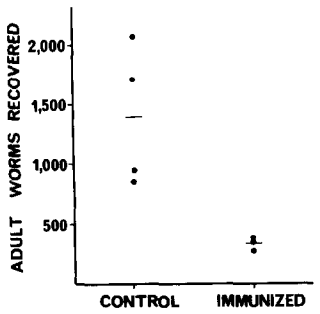

FIG. 2. Adult *Ancylostoma ceylanicum* worms recovered from individual animals in the control groups 6 weeks after infection.

The initial red cell mean corpuscular volumes in the control and immunized animals were 66.7 ± 1.0 and 69.1 ± 1.4 fl, respectively; this difference was not statistically significant. No significant change in the parameter was observed in either group for the duration of the infection.

The initial mean white cell counts in the control and immunized dogs were 14.6 ± 2.9 and 13.5 ± 2.0 × 10^9 cells per liter, respectively; this difference was not statistically significant. No significant change was noted in either group for the 6 weeks of observation.

The eosinophil levels in control and immunized dogs are indicated in Fig. 3. Both groups of animals developed a significant eosinophilia following infection, but there were no differences between the two groups.

The initial mean platelet counts for the control and immunized dogs were 380 ± 210 and 430 ± 80 × 10^9 platelets per liter, respectively; this difference was not statistically significant. No significant change was noted in either group for the duration of the infection.

The initial spontaneous lymphocyte transformations as measured by [^{3}H]thymidine uptake in the absence of mitogen or

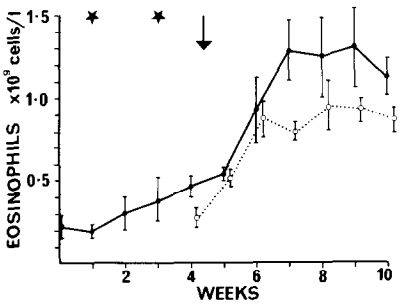

FIG. 3. Peripheral blood eosinophil counts in dogs immunized with *Ancylostoma ceylanicum* (●) and control (○) dogs at weekly intervals. The star denotes administration of an immunizing dose, and the arrow indicates the time of infection. Three dogs were in the immunized group and four dogs in the control group. Results are expressed as the means ± SEM.

antigen in control and immunized animals were 460 ± 100 and 370 ± 110 dpm, respectively; this difference was not statistically significant. Similarly, no significant differences were noted within or between the 2 groups for the period of observation.

The initial lymphocyte transformations induced by phytohemagglutinin in control and immunized dogs were 2200 ± 1700 and 7300 ± 3400 dpm, respectively; this difference was not statistically significant. Similarly, no differences were noted within or between the 2 groups of dogs for the duration of the experiment.

The initial stimulation indices of lymphocytes incubated with larval antigen for control and immunized dogs were 1.3 ± 0.6 and 0.6 ± 0.2 dpm, respectively; this difference was not statistically significant. The stimulation index is the ratio of dpm in the stimulated cultures to the dpm in the unstimulated (spontaneous) cultures. Larval antigen-induced stimulation was seen following immunization and infection in the immunized dogs; the stimulation indices immediately prior to infection, one and two weeks after infection were 1.3 ± 0.2 ($P < 0.025$), 2.2 ± 0.5 ($P < 0.02$), and 1.7 ± 0.8 ($P < 0.02$) dpm, respectively. There was no significant stimulation with this antigen observed in the control group during the 6 weeks of infection.

The initial stimulation indices of lymphocytes incubated with adult worm antigen from control and immunized dogs were 1.2 ± 0.6 and 0.7 ± 0.2 dpm, respectively; this difference was not statistically significant. Adult worm antigen-induced lymphocyte transformation was seen following infection. Significant stimulation was measured in the immunized group of dogs 1 week (1.6 ± 0.4, $P < 0.05$) and 2 weeks (2.3 ± 0.6, $P < 0.025$) after infection. There was no significant stimulation with this antigen in the control group during the 6 weeks of infection.

The serum antibodies of the IgM, IgG, and IgA classes to hookworm-derived antigens are shown in Fig. 4. IgM antibodies did not develop during immunization, but they did appear in both groups after infection. They first appeared 1 week after infection, reached a peak by the second week for the control group ($P < 0.01$) and the immunized group ($P < 0.05$), and then declined and disappeared by the sixth week after infection; there was no significant difference between the two groups prior to infection and at any time after infection.

IgG antibodies did not appear during immunization; however, they were first seen in the immunized dogs at the time of infection, were significantly elevated on the second week ($P < 0.05$), and continued to rise for the duration of the infection. Levels of IgG antibody in the control dogs did not increase until the fourth week ($P < 0.02$) of infection, after which they continued to rise for the duration of the infection. There was no significant difference in levels of IgG antibodies between immunized and control animals at the time of infection. Two, three, and four weeks after infection, however, IgG antibody titer of immunized dogs was significantly greater ($P < 0.05$, $P < 0.01$, and $P < 0.05$, respectively) than control animals; by 5 weeks after infection, there was

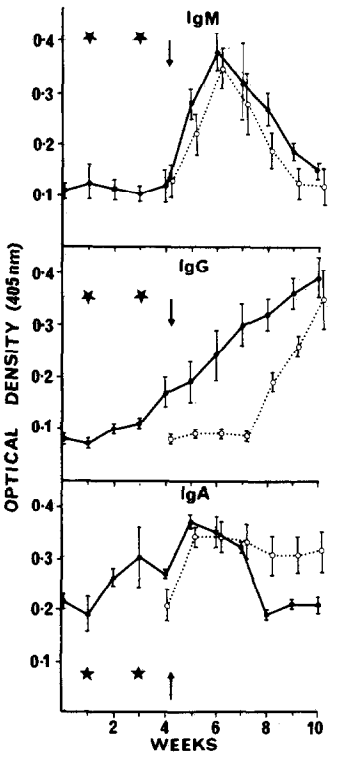

FIG. 4. Serum antihookworm IgM (upper), IgG (middle), and IgA (lower) antibody levels for dogs immunized with *Ancylostoma ceylanicum* (●) and control (○) dogs before and at various times after infection. The star denotes administration of an immunizing dose, and the arrow indicates the time of infection. Three dogs were in the immunized group and four dogs in the control group. Results are expressed as the means ± SEM.

no significant difference between the two groups of dogs.

Serum IgA antibodies did not develop significantly during simmunization; however, they did appear in both groups after infection. They were significantly elevated in the immunized dogs 1 week after infection ($P < 0.02$), were maintained at this level until the third week, and then declined rapidly. Four, five, and six weeks after infection, the level of this antibody in immunized animals was significantly less ($P < 0.05$, $P < 0.025$, and $P < 0.05$, respectively) than control dogs; IgA antibodies appeared in the control dogs 1 week after infection ($P < 0.05$) and remained at this level for the duration of the infection.

DISCUSSION

Three species of hookworm complete their life cycle in humans, namely, *Ancylostoma duodenale, Necator americanus,* and *A. ceylanicum.* The third species does, however, have a wider host range, infecting dogs, cats, and man. We have used this species of hookworm to establish a model of human ancylostomiasis and have shown that, like humans, dogs develop chronic infections (Carroll and Grove 1984). More recently, we have examined aspects of resistance to reinfection in dogs infected with *A. ceylanicum* and have observed that dogs with chronic hookworm infection are considerably more resistant to challenge 1 month after the termination of the primary infection with anthelmintics (Carroll and Grove a, in press). Subsequently, we have described the observation that dogs currently infected with small numbers of larvae are partially resistant to a challenge infection with a large number of infective larvae (Carroll and Grove b, in press). In both these simulations of infections in hookworm endemic areas, there were significant reductions in both the fecal egg excretion and the intestinal adult worm burden in the challenge animals. Likewise, we have shown in this study that dogs immunized with soluble antigen extracted from adult worms are partially resistant to challenge infection with large numbers of infective larvae. The similar reduction in the fecal egg excretion and intestinal adult worm burden indicates that this resistance is achieved by a decrease in adult worm burden rather than by inhibition of fecundity of adult female worms. The percentage reductions in fecal egg excretion and intestinal adult worm burden in dogs immunized with soluble antigen were slightly less than that seen in animals reinfected or superinfected with *A. ceylanicum,* which may indicate that the resistance ac-

quired from a natural infection is greater than that gained from the immunizing doses administered in this study.

In addition to measuring parasitological parameters, we compared a number of hematological and immunological responses in the immunized dogs with animals who had received a primary infection with the same number of larvae. In a previous study, dogs challenged during the tenure of a small primary infection did not develop anemia similar to the control animals when infected with 5000 larvae. Earlier, it was found that an infective dose of at least 10,000 larvae is necessary to produce marked microcytic anemia (Carroll and Grove 1984). In the present experiment, neither group of animals developed anemia when infected with 5000 larvae; therefore, it is not possible to say that the immunization protected the animals from hookworm disease.

Evidence that both groups of dogs produced immune responses is indicated by the appearance of specific antibodies, antigen-induced lymphocyte blastogenesis, and blood eosinophilia. IgM antibodies were not detected during immunization, and the transitory appearance of these antibodies after infection in the immunized animals was not significantly different to the control dogs; the reasons for this are uncertain. However, immunized dogs developed specific IgG antibodies to a fraction of adult worm antigen earlier than did control dogs indicating that they showed an anamnestic response following infection. Specific IgA antibodies were not stimulated during immunization, and following infection, levels of this antibody rose significantly in both groups of dogs. Four weeks after infection however, there was a decline in serum IgA antibody in the immunized animals. As this class of antibody is often associated with intestinal immunity, the lower adult worm burden of the immunized dogs may have been insufficient to stimulate sustained levels of this antibody. Alternatively, if the same number of larvae reached the gut in the immunized dogs as in the control dogs, the rapid decline in IgA levels may have coincided with the expulsion of worms from the intestine.

Although in previous studies we have shown that infection with *A. ceylanicum* results in transitory lymphocyte responsiveness four weeks after infection (Carroll and Grove 1984), larval and adult worm antigen-induced lymphocyte stimulation was only detected in the immunized animals. There were no differences between the two groups with respect to blood eosinophilia, which developed 2 weeks after infection and remained elevated for the duration of the infection. The relative roles of these responses as defense mechanisms in hookworm infection require further elucidation. In addition, different immunization regimens with repeated doses or greater amounts of antigen may induce other variations in immunological responses.

McCoy (1931) indicated that, under conditions of repeated infection, dogs acquired significant immunity to challenge infection with *A. caninum*. Later, in a series of experiments, Thorson (1956) demonstrated that injections of extracts of the esophagus of adult hookworms resulted in a significant reduction in adult worm numbers when compared with control animals eight days after the appearance of eggs in the feces. Extending this observation of acquired immunity in hookworm infection, Miller (1964) studied the reduction in infectivity of normal *A. caninum* larvae as measured by subsequent intestinal establishment of adult hookworms following a primary infection with X-irradiated larvae. Results of this work led to the development and commericalization of a vaccine against canine hookworm (Miller 1971). Miller showed that this type of vaccination protected dogs from hookworm disease by lowering the adult worm burden.

In hookworm endemic areas, reinfection often occurs after anthelmintic therapy (Cort *et al.* 1929); however, a number of

studies suggest that, although the prevalence of infection does not remain greatly reduced, the intensity of infection is often decreased considerably, suggesting the acquisition of partial immunity (Docherty 1926; Hill 1926). These observations in humans, together with the present study in dogs, provide a basis for confidence that, with proper selection and presentation of protective antigens, vaccines conferring significant resistance to reinfection may be developed.

ACKNOWLEDGMENTS

This study was supported by grants from the National Health and Medical Research Council of Australia and TVW Telethon Foundation of Western Australia. We are grateful to Mr. C. Henderson of the Research Centre for care and maintenance of animals.

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