Resistance of dogs to reinfestation with *Ancylostoma ceylanicum* following anthelmintic therapy

S. M. Carroll and D. I. Grove

Dept. of Medicine, University of Western Australia, Perth, Western Australia

Abstract

A model of human hookworm infection has been developed which shows that dogs with chronic hookworm infection are considerably resistant to reinfection one month after the termination of the primary infection with anthelmintics. Challenge and control dogs were infected with 1,800 larvae and the infection was followed for six weeks. When compared with control dogs, faecal egg excretion and intestinal adult worm burdens in challenge dogs were reduced by 85% and 77%, respectively. Infection had no significant effect on haemoglobin concentrations, total white cell counts, platelet levels or spontaneous and phytohaemagglutinin-induced lymphocyte transformations in both control and previously infected dogs. Both groups of dogs developed an eosinophilia and lymphocytes responded transiently to stimulation with both larval and adult worm antigens, although there were no significant differences between the two groups of animals. Specific IgM antibodies were transient in both groups of animals following infection. Specific IgG antibodies were present at high levels before infection in challenge dogs when compared with control dogs, and fell transiently after challenge; three weeks after infection, IgG antibodies appeared in the control animals and titres continued to rise during the period of observation. Challenge dogs also developed specific IgA antibodies three weeks after infection, and these remained at high levels, but these antibodies were not detected in control dogs. Thus, dogs infected with this strain of the hookworm, *Ancylostoma ceylanicum*, which has been shown to infect man, develop functional protective immunity. These findings improve prospects for vaccine development.

Introduction

Hookworm is one of the most important human helminth infections, infecting almost one thousand million people (Peters & Gilles, 1977). Potential measures for the control of this infection include environmental sanitation and health education, mass administration of anthelmintics and immunization (Grove, 1982). Unfortunately, environmental sanitation is extremely costly and unlikely to provide significant benefits in most developing countries in the foreseeable future. Similarly, although effective anthelmintics are available, mass administration is often followed by a return to the original prevalence and intensity of infection (Bhai Bulaya et al., 1977), thus requiring repeated administration of drugs with all its attendant costs and logistic difficulties. Furthermore, there is always the risk that these practices may result in widespread drug resistance.

An alternative approach is the development of vaccines, particularly if they are able to induce long-lived immunity. This is more likely to be successful if natural infections confer some resistance to reinfection. Whether this occurs or not in man is controversial (Banwell & Schad, 1978; Miller, 1979). We have recently developed an animal model using one of the three hookworms that infect man, namely *Ancylostoma ceylanicum* (see Carroll & Grove, 1984). We have used this system to determine whether dogs whose chronic hookworm infections were terminated with anthelmintic administration, were resistant to challenge. In addition, we have measured the associated haematological and immunological responses to reinfection.

Materials and Methods

Parasite

*A. ceylanicum* was obtained originally from a dog in 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 worm has been shown to produce patent infections in man (Carroll & Grove, 1985) and has been passaged subsequently back to dogs. The techniques for measuring faecal egg excretion, intestinal adult worm burdens, haematological parameters and lymphocyte transformations have been described earlier (Carroll & Grove, 1984).

Serum antibodies to hookworm

Serum anti-hookworm 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 for *Strongyloides ratti* (see Carroll et al., 1981). Larval extract antigen was used for IgM antibody detection and partially purified antigens from adult worms, D1 and D2, were used for IgG and IgA anti-hookworm antibody detection, respectively; these antigens are fractions of adult worm antigen which are separated by Sephadex G200 column chromatography. Preliminary tests had shown these fractions were the most discriminatory in measuring IgG and IgA antibody activity. Microtitre trays (Linbro®; Flow Laboratories Inc., McLean, Virginia, 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 one hour at 37°C and then overnight at 4°C. All test sera were diluted 1:50 in wash buffer (0.1M phosphate buffer with 0.3% Tween 20) containing 10% foetal bovine serum, plates were washed using an automatically plate washer (Multwash®; Skatron AS, Lier, Norway) and all incubation steps were for one hour at 37°C. IgM and IgG antibodies were measured using optimally diluted goat anti-dog IgM and IgG antisera, respectively (Cappel Laboratories, Cochranville, Pennsylvania, USA) followed by alkaline phosphatase-labelled swine anti-goat IgG antibody (Tago Inc., Burlingame, California, USA) whereas specific IgA antibody was measured with rabbit anti-dog IgA antibody (Pel-Freeze Biologicals, Rogers, address: For correspondence: S. M. Carroll, Department of Medicine, University of Western Australia, Queen Elizabeth II Medical Centre, Nedlands, 6009, Western Australia.)
Arkansas, USA) followed by alkaline phosphatase-labelled goat anti-rabbit IgG antibody (Tago Inc.). Colour development using p-nitrophenol (1:0 mg/ml) phosphate was stopped after one hour with 1N NaOH and the optical densities read at 405 nm using an automated plate reader (Titretek Multiscan; Flow Laboratories Inc.).

**Animals**

Male mongrel dogs were obtained from the general public, housed and prepared as detailed elsewhere; dogs were treated with the anthelmintics pyrantel pamoate and buna-midine hydrochloride four weeks before infection (CARROLL & GROVE, 1984). Four dogs which had been infected with 2,000 larvae 24 weeks earlier, were treated with pyrantel pamoate (14 mg/kg) orally. Subsequently, no hookworm ova were found on examination of the stools. One month after treatment, these dogs together with four control dogs were infected with 1,800 larvae. Blood samples were obtained at 10 a.m. weekly and faeces were collected weekly. Six weeks after infection the animals were killed and adult worms in the intestines removed, sexed and counted.

**Statistics**

All results are expressed as mean ± standard deviation. All tests of significance were performed using the two-tailed Student's "t" test unless otherwise indicated.

**Results**

**Worm Burdens**

*Faecal egg excretion:* Eggs were first seen in the stools three weeks after challenge infection. Three, four, five and six weeks after infection, the reduction in faecal egg excretion in reinfected dogs compared with control animals was 86%, 88%, 83% and 87% respectively. These reductions were statistically significant (Fig. 1).

*Adult worm burden:* Dogs were examined at autopsy six weeks after challenge infection. There was a 77% reduction in adult hookworm numbers in reinfected dogs compared with control animals being 699 ± 376 and 163 ± 190 worms for the control and reinfected dogs, respectively (Fig. 2); these values represent recoveries of 39% and 9% of the infecting dose, respectively. The difference in adult worm burden was statistically significant (P<0.05, single tail). Since the worm burdens in control dogs followed a normal distribution while the distribution in reinfected dogs was log-normal, the data were recalculated using logarithms; the difference in worm burdens was again statistically significant (P<0.025, single tail). The mean percentages of female worms were 57 ± 2% and 62 ± 9% in the control and reinfected dogs respectively; this difference was not significant.

**Haematological Responses**

*Haemoglobin concentration:* There were no significant differences in haemoglobin concentrations between control and challenge dogs before challenge, the values being 16.1 ± 1.4 and 16.5 ± 0.8 g/dl respectively. Following reinfection, haemoglobin concentrations fell in both groups (minimum value 14.3 ± 0.9 g/dl), but there were no significant differences between the two groups.

*Mean corpuscular volume:* The initial red cell mean corpuscular volumes in control and challenge dogs were 69.9 ± 1.7 and 68.0 ± 1.8 fl respectively; this difference was not statistically significant. No significant change was noted in either group for the six weeks of observations.

**White cell counts:** The initial mean white cell counts in control and challenge dogs were 13.7 ± 3.3 and 8.9 ± 1.8 × 10⁶ cells per litre, respectively; this difference was not statistically significant. No significant change was noted in either group for the six weeks of observation.

**Eosinophil counts:** The eosinophil levels in control and reinfected dogs are indicated in Table I. Both groups of dogs developed a significant eosinophilia following infection, but there were no significant differences between the two groups.
Table I—Mean eosinophil counts in control and challenge dogs before and at weekly intervals after infection. There were four animals in each group.

<table>
<thead>
<tr>
<th>Weeks after infection</th>
<th>Eosinophils ($\times 10^9/\ell$)</th>
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<tbody>
<tr>
<td></td>
<td>Control x ± S.D.</td>
</tr>
<tr>
<td>0</td>
<td>320 ± 230</td>
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<tr>
<td>1</td>
<td>520 ± 110</td>
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<tr>
<td>2</td>
<td>740 ± 440</td>
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<tr>
<td>3</td>
<td>2000 ± 620*</td>
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<tr>
<td>4</td>
<td>1350 ± 780</td>
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<tr>
<td>5</td>
<td>1770 ± 480*</td>
</tr>
<tr>
<td>6</td>
<td>1500 ± 560*</td>
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* = P<0.5 when compared with week 0.

Platelet counts: The initial mean platelet counts in the control and reinfeected dogs were 263 ± 31 and 248 ± 26 x 109 platelets per litre respectively; this difference was not statistically significant. No significant change was noted in either group for the six weeks of observation.

Immunological Responses

Spontaneous lymphocyte transformation: The initial lymphocyte transformation as measured by $^3$H thymidine uptake in the absence of antigen or mitogen in control and chronically infected dogs were 770 ± 400 and 700 ± 340 disintegrations per minute (dpm) respectively; this difference was not statistically significant. Similarly, no significant differences were seen between the two groups of animals for the six weeks of observation.

Phytohaemagglutinin-induced lymphocyte transformation: The initial lymphocyte transformations induced by phytohaemagglutinin in control and reinfected dogs were 9,900 ± 4,100 and 23,000 ± 22,000 dpm, respectively; this difference was not statistically significant. Similarly, no significant differences were seen between the two groups of dogs for the duration of the experiment.

Antigen-induced lymphocyte transformation: The initial stimulation indices of lymphocytes incubated with larval antigen from control and challenge dogs were 1·2 ± 0·4 and 1·4 ± 0·4, respectively; this difference was not statistically significant. Larval antigen-induced lymphocyte stimulation was seen following infection; the maximal indices were four weeks after infection and were 2·9 ± 0·8 (P<0·02) and 3·6 ± 4·2 for control and reinfected dogs, respectively. There was, however, no statistical difference between the two groups of dogs during the six weeks of observation.

The initial stimulation indices of lymphocytes incubated with adult worm antigen from control and reinfeected dogs were 0·9 ± 0·3 and 0·8 ± 0·6, respectively; this difference was not statistically significant. Adult worm antigen-induced lymphocyte transformation was seen following infection. Significant stimulation was seen for control dogs with a primary infection four weeks after infection (3·0 ± 1·0, P<0·02) and five weeks after infection for reinfeected dogs (2·4 ± 0·9, P<0·05). There was, however, no statistical difference between the two groups of dogs during the six weeks of observation.

Serum antibody levels: The serum anti-hookworm antibodies of the IgM, IgG and IgA classes are shown in Fig. 3. IgM antibodies appeared transiently. They were first seen one week after infection, reached a peak level by the second week, then declined and disappeared by the fourth week after infection. There was no significant difference in the IgM antibody levels between the two groups of dogs before infection and following infection there were no significant differences in the changes in optical densities between the two groups.

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Fig. 3. Serum anti-hookworm IgM (upper panel), IgG (middle panel), and IgA (lower panel) antibody levels for control (○—○) and challenge (■—■) dogs before and at various times after infection. Four dogs were in each group. Results are expressed as the mean ± S.E.
The titre of IgG anti-hookworm antibody of the reinfected dogs was, however, significantly greater than the control dogs before infection (P<0·001). Following infection the titre of this antibody in the reinfected group declined significantly by two weeks (P<0·05) and then increased rapidly to a level higher than before infection. The IgG antibody levels in the control dogs rose rapidly during the third week of infection (P<0·001); by six weeks after infection there was no significant difference between the two groups of dogs.

Before infection and for the two weeks following there were no significant differences in IgA anti-hookworm antibody levels between the two groups of dogs. However, the specific IgA antibody levels in the reinfected group increased three weeks after infection; this increase was significant four, five and six weeks after infection (P<0·05). No significant change in this parameter was observed in the control animals at any time during the infection.

**Discussion**

**GILMAN** (1982) has deplored the absence of a suitable model of human hookworm infection which would allow assessment of the acquisition of functional protective immunity. We have developed such a model and have now shown clearly that dogs with chronic hookworm infection are considerably resistant to challenge one month after termination of the primary infection with anthelmintics. The reduction in faecal egg excretions and intestinal worm burdens were both of the order of 80%, thus indicating that previous exposure did not alter the fecundity of those hookworms which reached maturity. There are two possible reasons for the failure of worms to mature. Either non-specific inflammation renders the gut an unfavourable environment for intestinal worms or the acquisition of immunological memory permits the immune defences to become active against either larvae migrating through the tissues or worms in the bowel. The latter seems more likely, since we allowed one month after termination of the original hookworm infection before reinfection in order to allow any intestinal inflammation to subside.

The results we obtained in dogs infected with this human parasite are similar to those found with the dog hookworm, *A. caninum*, whether following a natural primary infection or after exposure to attenuated, irradiated larvae (MILLER, 1964; VINEAYAK et al., 1981). The implications of this finding are enormous. It is generally agreed that the likelihood for the development of hookworm disease is dependent upon the worm burden (MILLER, 1979; VARAYAN & BANWELL, 1982) and we have confirmed that this is also the case in our system (CARROLL & GROVE, 1984). Thus, a reduction in worm burden, and hence blood loss, of 80% is likely to be sufficient to protect the host from hookworm anaemia unless there is severe concomitant malnutrition.

In addition to examining the maturation of hookworm in reinfected dogs, we compared a variety of haematological and immunological responses in these animals with dogs who had received a primary infection with the same infective dose. Neither group of dogs developed hookworm anaemia, but this was not surprising since we have found earlier that an infective dose of at least 10,000 larvae is necessary to produce marked microcytic anaemia (CARROLL & GROVE, 1964). Both groups of animals evinced evidence of immune responsiveness as indicated by the appearance of antibodies, antigen-induced lymphocyte stimulation and blood eosinophilia. The transient appearance of the ELISA-larval antigen IgM antibodies in control dogs with a primary infection was similar to that observed in animals infected with *A. ceylanicum* and detected by immunofluorescence using living filariform larvae (CARROLL & GROVE, 1984), and no significant anamnestic effect was seen in challenged animals. Reinfected animals demonstrated significantly greater IgG antibody activity to the adult worm antigen before infection than the control animals. This fell transiently after re-exposure to *A. ceylanicum* and may indicate consumption of antibody by antigen. The fact that this occurred during the phase of systemic migration before the appearance of patent infection suggests that IgG antibodies may enhance the host's resistance to larvae in the tissues. A marked difference was seen between the two groups of dogs with respect to serum IgA antibody levels. These antibodies appeared only in reinfected animals. Although it is not clear how much they reflect the concentrations of IgA in the bowel, these findings are consistent with the concept that these antibodies may play a role in the prevention of functional immunity against the intestinal phase of the parasite. Even though there were no differences between the two groups of dogs with respect to antigen-induced lymphocyte stimulation and blood eosinophilia, these parameters were both altered by infection and probably both play a part in the genesis of resistance to infection. The relative roles of these various defence mechanisms requires further investigation.

In his recent review, MILLER (1979) emphasized the need for evidence pertaining to the development of functional protective immunity in man infected with hookworm. Despite some evidence to the contrary (BRUMPT, 1952; BALL & BARTLETT, 1969), he was of the view that immunity in man is likely to be very similar to that observed in the dog -*A. caninum* system. We have now partially bridged the gap between these two host-parasite relationships by demonstrating that *A. ceylanicum*, a human parasite, stimulates considerable resistance to reinfection in dogs. This increases the likelihood that a similar effect may be found in man with hookworm infection and improves the prospect for the ultimate development of a vaccine.

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**References**


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