Response of dogs to challenge with *Ancylostoma ceylanicum* during the tenure of a primary hookworm infection

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Abstract

A model of human hookworm infection has been developed which shows that dogs currently infected with small numbers of hookworms are considerably resistant to a challenge infection with a large inoculum of infective larvae. Two groups of dogs were infected with 500 larvae and four weeks after infection one group together with a previously uninfected control group were infected with 5,000 larvae and followed for six weeks. When compared with the secondary infection control dogs, faecal egg excretion and adult worm burdens were reduced by an average of 83 and 78% respectively. Infections had no significant effect on total white cell counts, platelet levels or spontaneous, phytohaemagglutinin- and antigen-induced lymphocyte transformation among the three groups of dogs. Dogs previously uninfected with hookworm developed a marked anaemia when infected with 5,000 larvae but this was not observed in the superinfected group. An eosinophilia developed in all groups and there were no significant differences among the three groups of animals. Specific IgM antibodies developed transiently in all groups of dogs two weeks after infection. IgG antibody levels were significantly greater in the superinfected animals one, two and three weeks after challenge infection compared with the secondary infection control animals; by four weeks there was no significant difference between the two groups of animals. Both groups given the large inoculum of larvae developed specific IgA antibodies one week after the challenge infection and these continued to rise in the superinfected group until termination of the experiment. It is concluded that dogs currently infected with the hookworm, *Ancylostoma ceylanicum*, demonstrate the development of functional protective immunity.

Introduction

In previous studies, we have developed an animal model of human hookworm infection by infecting dogs with *Ancylostoma ceylanicum*. As in man, these dogs developed chronic infections (CARROLL & GROVE, 1984) and the severity of disease was proportional to the worm burden. Following termination with anthelmintics of chronic hookworm infection, considerable resistance to reinfection was observed, with both faecal egg excretion and intestinal worm burdens being reduced by approximately 80% in challenged animals (CARROLL & GROVE, 1985a).

In hookworm endemic areas, people are usually exposed to infection repeatedly. It is therefore necessary to determine if current infection confers resistance to a second infection. We infected a group of dogs with small numbers of hookworms, then challenged half of them and a previously uninfected control group with large numbers of larvae. The influences of current infection on changes in worm burdens together with haematological and immunological responses following superinfection were evaluated.

Materials and Methods

*A. ceylanicum* was obtained originally from an infected dog in Malaysia (CARROLL et al., 1983); it has since been passaged through dogs, cats and man. The methods of preparation of the parasite and techniques of measuring faecal egg excretion, intestinal worm burden, haematological parameters and lymphocyte transformations were as described earlier (CARROLL & GROVE, 1984) with the following modification. In lymphocyte transformation studies, viable lymphocytes were diluted to 1.75 × 10⁶ cells/ml before addition to the trays and cells were incubated for four days before pulsing with ³H-thymidine. The ELISA for measuring serum anti-hookworm antibody levels has been detailed elsewhere (CARROLL & GROVE, 1985b).

Male mongrel dogs were obtained, housed and prepared for percutaneous infection as described previously (CARROLL & GROVE, 1984). Their ages ranged between three and five months and the median weight was 9.1 kg. They were divided as evenly as possible into three groups of four dogs each. The experimental design with the sizes of the infective doses and the times of infection for each of the groups of animals is outlined in Fig. 1. One animal in group C, however, became ill during the experiment just before challenge infection and was removed from the study. Faeces were collected weekly and blood samples were obtained at 10 a.m. on the same day. Ten weeks after commencement of the study, dogs were killed, the intestines opened longitudinally and adult worms removed, sexed and counted.

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

No significant differences were seen between groups A and B, given a primary infection of 500 larvae, both before and after superinfection of group B with 5,000 larvae. When group B was compared with group C given a control infection of 5,000 larvae, however, there was a significant reduction in faecal egg excretion three, four, five and six weeks (P < 0.05, < 0.025, < 0.025, single tail, respectively).

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after infection (Fig. 2). Statistical significance was achieved despite retention of the component of egg excretion resulting from the original infection of group B with 500 larvae; if it had been possible to remove this contribution mathematically, the statistical significance would have been even greater.

**Adult worm burden**

The number of adult worms recovered from the three groups of dogs is shown in Fig. 3. There was no significant difference between groups A and B, the adult worm burdens being 136 ± 116 and 550 ± 340, respectively. When group B was compared with

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**Table 1: Experimental Design**

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<td>C</td>
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**Fig. 1.** Experimental design: details of groups of animals, size of infective doses and times of infection.

**Fig. 2.** Faecal egg excretion of dogs in groups A (○○○), B (●●●) and C (△△△△△) at various times after infection. The arrow (→) indicates time of secondary infection. Four dogs were in each of the groups A and B, and three dogs in group C. Results are expressed as mean ± S.E.
group C \((1710 \pm 780)\), however, a statistically significant reduction was found \((P<0.05, \text{ single tail})\), even though the contribution of worms from the original infection was not removed. When this was done, there was a 78\% reduction in adult worm burden in superinfected dogs. The mean percentage of female worms in groups A, B and C were 59 \pm 5, 70 \pm 2 and 61 \pm 6, respectively.

### Haematological Parameters

#### Haemoglobin concentration

There were no significant differences among the three groups of dogs either before infection or four weeks after infection of groups A and B. Three weeks after infection of groups B and C with 5,000 larvae, however, there was a significant fall in haemoglobin concentration in group C \((P<0.025, \text{ paired } ^*t* \text{ test})\) but no significant change in group B, when compared with the values obtained at the time of superinfection (Fig. 4).

#### Mean corpuscular volume

There were no significant differences among the three groups in initial red cell corpuscular volumes; the mean values for groups A, B and C being 64 \pm 4, 66 \pm 3 and 66 \pm 3 fl., respectively. No significant change developed either within groups or among groups following infection.

#### White cell counts

There were no significant differences among the three groups of dogs in initial white cell counts. The mean values for groups A, B and C were 11.9 \pm 4.0, 11.2 \pm 2.1 and 8.8 \pm 1.1 \times 10^9 \text{ cells per litre}, respectively. No significant change developed among the groups following infection.

#### Eosinophil counts

There were no significant differences in initial blood eosinophil counts among the three groups of dogs. An eosinophilia developed in groups A and B following infection with 500 larvae. Similarly, an eosinophilia developed in group C following infection with 5,000 larvae but there was no significant further increase in blood eosinophil levels in group B challenged with the same number of larvae (Fig. 5).

#### Platelet counts

The initial mean platelet counts in the three groups of dogs were 300 \pm 130, 300 \pm 90 and
Fig. 5. Peripheral blood eosinophil counts in dogs of groups A (○—○), B (●—●) and C (△—△) at various times after infection. Four dogs were in each of the groups A and B, and three dogs in group C. The arrow (→) indicates the time of the secondary infection. Results are expressed as mean ± S.E.

Immunological Responses

Spontaneous lymphocyte transformation

The initial spontaneous lymphocyte transformations as measured by $^3$H-thymidine uptake in the absence of mitogen or antigen in groups A, B and C were 460 ± 320, 320 ± 210 and 430 ± 340 disintegrations per minute (d.p.m.), respectively. There were no significant differences among the three groups both before and at any time after infection.

Phytohaemagglutinin-induced lymphocyte transformation

The initial lymphocyte transformations induced by phytohaemagglutinin in groups A, B and C were 25,000 ± 17,000, 19,000 ± 18,000 and 27,000 ± 23,000 d.p.m., respectively. There were no significant differences among the three groups before or at any time after infection.

Antigen-induced lymphocyte transformation

The initial stimulation indices of lymphocytes incubated with larval antigen in groups A, B and C were 1.3 ± 1.6, 1.4 ± 1.8 and 1.9 ± 0.8, respectively. There were no significant differences among the three groups both before or at any time after infection. The initial stimulation indices of lymphocytes incubated with adult worm antigen in groups A, B and C were 280 ± 50 × 10^9 platelets per litre for groups A, B and C, respectively. There were no significant differences either within the groups or among the groups both before and at any time after infection.
1.0 ± 0.5, 1.6 ± 0.6 and 2.0 ± 0.8, respectively. There were no significant differences among the three groups before or at any time after infection.

**Serum antibody levels**

The serum antibody levels of the IgM, IgG and IgA classes to hookworm-derived antigens are shown in Fig. 6. IgM antibodies appeared transiently following infection; they were elevated slightly two weeks after infection with 500 larvae in groups A and B and elevated significantly two weeks after administration of the challenge infection of 5,000 larvae to groups B (P<0.02) and C (P<0.05). In group B, however, there was no significant difference between the IgM responses to primary and challenge infection.

IgG antibodies first appeared four weeks after primary infection in group A and were elevated significantly six weeks after infection (P<0.005); they remained at this level for the duration of the infection. Similarly, IgG antibodies were also elevated significantly (P<0.005) in group B four weeks after primary infection with 500 larvae. By one week after challenge infection, the level of IgG antibody in group B was significantly greater (P<0.02) than the superinfection control group C and remained so two (P<0.05) and three (P<0.01) weeks after challenge. Four weeks after the challenge infection of 5,000 larvae, specific IgG antibody levels rose in the previously uninfected control group C (P<0.05) and by six weeks after infection there was no significant difference among the three groups of dogs with respect to IgG antibody levels.

IgA antibodies were not detectable in groups A and B during the tenure of the infection with 500 larvae. IgA antibody levels began to rise in groups B and C one week after challenge infection with 5,000 larvae. This rise was statistically significant (P<0.025) in group C at this time when compared with the level immediately before infection and did not alter significantly thereafter. The rise in group B animals did not become significant (P<0.05) until two weeks after challenge infection but in this group antibody levels continued to rise until the termination of the infection.

**Discussion**

We have shown clearly that dogs currently infected with small numbers of hookworms are partially resistant to a challenge infection with a large inoculum of infective larvae. The degree of resistance achieved in this experiment in which dogs were infected for only a short period was very similar to that when dogs with long standing infections were treated with anthelmintics then challenged.

The reductions in faecal egg excretion and intestinal adult worm burden in superinfected dogs were similar. This indicates that reduced egg output was achieved by a decrease in intestinal adult worm numbers rather than by inhibition of fecundity of adult female worms. McCoy (1931) found when studying *A. caninum* in dogs under conditions of repeated infection with heavy doses of larvae that the egg count dropped suddenly with passage of large numbers of adult worms. Similarly, Okada (1931) reported a reduction in the numbers of worms in the gut of adult dogs following superinfection with *A. caninum*. These findings have significant implications for protection against the development of hookworm disease since its severity is related to increasing worm burden (Carroll & Grove, 1984). This has been substantiated by a finding that superinfected dogs with small worm burdens did not develop the anaemia that was seen in the control animals given a primary infection of 5,000 larvae.

The mechanisms by which these superinfected animals resist infection are obscure. There may be either inhibition of maturation of larvae or accelerated expulsion of adult worms from the gut. Our measurement of various immunological parameters helped only marginally in defining the roles of humoral and cell-mediated immunity in the genesis and implementation of this resistance. In previous studies, we have shown that dogs infected with *A. ceylanicum* show transient lymphocyte responsiveness to both larval and adult hookworm antigens about four weeks after infection (Carroll & Grove, 1984). In studies of human patients infected with *A. ceylanicum*, we found that greater stimulation indices were achieved when lymphocytes were incubated in lower concentrations and for longer periods (Carroll & Grove, in press). We adopted this protocol in the present study but unfortunately we failed to demonstrate any significant lymphocyte stimulation by hookworm antigens.

As expected, there were transient IgM responses to infection and the development of long-lasting high titres of IgG antibodies. It is noteworthy that IgA antibodies were not detectable in the serum of animals given the light infection of 500 larvae but were found in those given a heavy infection of 5,000 larvae. The relationship between serum and intestinal IgA levels is uncertain but these findings suggest that the size of the IgA response may be dependent upon the magnitude of the hookworm burden. Since it is possible that IgG antibodies are involved in the genesis of resistance to larvae migrating systemically and that IgA antibodies are concerned with immunity to developing or adult worms in the gut, it would be of considerable interest to measure specific intestinal IgA production in response to hookworm infection.

Epidemiological evidence indicates that the prevalence and intensity of hookworm infection in man does not increase with advancing years (Banwell & Schad, 1979). It is uncertain whether this is due to changing behaviour and hygiene of older people or whether it reflects the partial acquisition of resistance to reinfection. The data provided in this animal model suggest that specific immunity may be acquired and auger well for the development of a vaccine as a possible interventionist strategy against this parasite.

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**References**


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