EXPERIMENTAL INFECTION OF HUMANS WITH
ANCYLOSTOMA CEYLANICUM: CLINICAL, PARASITOLOGICAL,
HAEMATOLOGICAL AND IMMUNOLOGICAL FINDINGS

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Abstract. Experimental infection of two human volunteers with Ancylostoma ceylanicum produced patent infection. Following percutaneous infection with 1200 infective larvae, there was no evidence of skin eruption, however, severe abdominal discomfort was experienced by both subjects coincidently at various intervals. Eggs appeared in the faeces five weeks after infection; low level excretion continued until termination of the infection 30 weeks after inoculation. Infection had no significant effect on haemoglobin concentrations, total white cell counts, platelet levels or spontaneous and phytohaemagglutinin-induced lymphocyte transformations in either of the infected volunteers. Persistent eosinophilia began four weeks after infection in both infected persons; lymphocytes from one subject responded transiently to stimulation with larval antigen while lymphocytes from both infected subjects responded to stimulation with adult worm antigen. Specific IgM and IgG antibodies appeared two and six weeks after infection, respectively, and persisted for the duration of the experiment.

Key words: Ancylostoma ceylanicum; patent infection; human hookworm.

Introduction

Hookworm infection is a major cause of morbidity in less developed countries with nearly one billion people being infected [1]. Iron deficiency anaemia is the most important clinical manifestation of the disease, often resulting in a decrease in worker productivity [2]. Gastrointestinal symptoms have been difficult to delineate because of frequent intercurrent infection with other gut pathogens. Three species of hookworm infect humans; Ancyllostoma duodenale, Necator americanus and A. ceylanicum. The first two species only infect humans whereas A. ceylanicum has a wider host range infecting dogs, cats and man [3].

There have been few descriptions of experimental infections of humans with A. ceylanicum [4–7]. A. braziliense and A. ceylanicum were considered synonymous for many years until they were separated by Biocca [8] and resolved definitively by cross breeding experiments [9]. In all the studies referred to above, A. ceylanicum was almost certainly the parasite investigated.

We undertook the following experiment in order to determine whether the strain of
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*A. ceylanicum* which we have obtained from Malaysia [10] produced patent infection in humans. In this paper, we report clinical, parasitological, haematological and immunological observations in two human volunteers infected with this parasite.

**Materials and Methods**

*Parasite.* *A. ceylanicum* was obtained originally from an infected dog in Malaysia. The acquisition of the worm and maintenance of the lifecycle have been described in detail earlier [10]. Percutaneous infection was facilitated by the application of 1200 infective larvae suspended in 0.05 ml phosphate buffered saline (PBS) to the inner surface of the forearm of each of the two subjects. Venous blood was obtained from the forearm of each of the infected subjects and two control subjects at 9.00 a.m. and faeces were collected before and at various intervals after infection.

*Parasitological parameters.* In order to measure faecal egg excretion, 1 gm aliquots of faeces were weighed, broken up in a small quantity of water in 15 ml centrifuge tubes, and suspended in 10 ml of saturated sodium chloride solution and eggs were quantified in Whitlock chambers [11]. Following 30 weeks of observation, the infection was terminated by the oral administration of pyrantel pamoate (10 mg/kg).

*Haematological and immunological parameters.* The techniques for measuring haematological and immunological parameters were as described earlier [12] with the following modifications. In lymphocyte transformation studies, viable lymphocytes were diluted to 1.75 X 10^6 cells/ml and cells were incubated for four days prior to pulsing with \(^3\)H-thymidine. Preliminary experiments made with a range of both mitogen and antigen indicated that lymphocytes responded to stimulation when incubated with 20 ug per well of phytohaemagglutinin (PHA, Bacto-phytohemagglutinin P; Difco Laboratories, Detroit, Michigan, USA) and when incubated with 10 ug per well of both soluble larval and adult worm antigens.

Serum anti-hookworm antibody levels were measured by an enzyme-linked immunosorbent assay similar to that described elsewhere [13] with certain modifications. 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 were separated by Sephadex-G200 column chromatography. Microtitre trays (Linbro<sup>®</sup>; Flow Laboratories Inc., McLean, Virginia, USA) were coated with 10 ug/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 (0.1 M phosphate buffer with 0.05% Tween 20) containing 10% foetal bovine serum, plates were washed using an automated plate washer (Multiwash<sup>®</sup>, SkatronAS, Lier Norway) and all incubation steps were for one hour at 37°C. IgM, IgG and IgA antibodies were measured using optimally diluted alkaline phosphatase-labelled goat anti-human IgM, IgG and IgA antisera, respectively (Tago Inc., Burlingame, California, USA). Colour development using p-nitrophenol phosphate (1 mg/ml) was stopped after one hour with 1 N NaOH and the optical densities read at 405 nm using an automated plate reader (Titretek<sup>®</sup> Multiscan; Flow Laboratories Inc.).

**Results**

*Clinical features.* Following the application of infective larvae to the skin, there was no evidence of any skin reaction in either subject. Twenty nine days after infection, one subject experienced an abrupt onset of frequent severe colicky abdominal pains associated with diarrhoea and excessive flatulence which disabled him for 24 hours. This was followed by low grade abdominal distension and discomfort for one week. The other infected person developed on the same day similar but less marked symptoms which persisted for one week. Both infected persons experienced a mild recurrence of these symptoms 45 days after infection; similarly lasting for one week. Fifty seven days after infection, both individuals again had mild abdominal discomfort and distension for several days. This was followed by asynchronous attacks of decreasing severity in both subjects over the next few months.
Faecal egg excretion. Eggs were first noted in the faeces five weeks after infection. Chronic low grade excretion of eggs continued until termination of the infection with an anthelmintic 30 weeks after infection (Figure 1). Following termination, eggs were not found in the faeces of either of the previously infected subjects.

Blood picture. Marked effects were not seen in infected subjects with respect to haemoglobin concentration, red cell mean corpuscular volume, total white cell count or platelet count.

Blood eosinophil counts. The blood eosinophil counts for each person are illustrated in figure 2. Levels remained relatively constant in the two uninfected control subjects but a marked eosinophilia developed in the two infected persons, beginning about four weeks after infection, the levels of eosinophils declined and four weeks later were similar to the levels prior to infection.

Lymphocyte transformations. The spontaneous uptake of $^3$H-thymidine by lymphocytes in the absence of mitogen or specific antigen before infection was 660 and 2300 disintegrations per minute (d.p.m.) in the two subsequently infected subjects, and 460 and 1500 d.p.m. in the two control subjects. No marked variation in spontaneous uptake occurred in any person at any time after infection.

The PHA-induced uptake of $^3$H-thymidine by lymphocytes prior to infection was 130,000 and 210,000 d.p.m. in the two subsequently infected persons, and 190,000 and 230,000 d.p.m. in the two control subjects. No marked variation was observed in any subject at any time after infection.

Profound differences in hookworm antigen-induced stimulation of lymphocytes were noted between the two groups of subjects. When lymphocytes were incubated with larval antigen, one infected subject had transitory marked stimulation three weeks after infection (Figure 3). No such stimulation was noted in the other infected persons or in the uninfected persons. When lymphocytes were incubated with adult worm antigen, marked responses were seen in cells obtained from both infected persons but were not found in those from the control subjects (Figure 4).
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Figure 2. Eosinophil numbers in the peripheral blood for infected subject 1 (■ ■ ■) and subject 2 (• • •), and the two uninfected controls (▽-▽, △-△) at various times after infection. The arrow (---) indicates time of anthelmintic treatment.

Figure 3. Stimulation indices of lymphocytes from infected subject 1 (■ ■ ■) and subject 2 (• • •), and the two uninfected controls (▽-▽, △-△) incubated with larval antigen at various times after infection. The stimulation index is the ratio of d.p.m. in the stimulated cultures to the d.p.m. in the unstimulated (spontaneous) cultures. The arrow (---) indicates time of anthelmintic treatment.
Figure 4. Stimulation indices of lymphocytes from infected subject 1 (■—■) and subject 2 (●—●), and the two uninfected controls (△—△) incubated with adult worm antigen at various times after infection. The stimulation index is the ratio of d.p.m. in the stimulated cultures to the d.p.m. in the unstimulated (spontaneous) cultures. The arrow (→) indicates time of anthelmintic treatment.

Figure 5. Serum anti-hookworm IgM (upper panel) and IgG (lower panel) antibody levels for infected subject 1 (■—■) and subject 2 (●—●) at various times after infection. Optical densities of control subjects were at all times less than 0.1 and 0.05 for IgM and IgG assays, respectively. The arrows (→) indicate time of anthelmintic treatment.
whom response was seen with larval antigen, showed profound stimulation with adult worm antigen by three weeks after infection. This declined slowly over the next 10 weeks. The stimulation index in the other infected person rose progressively to a peak six weeks after infection, then declined slowly. Four weeks after the termination of the infection, there were no differences in the stimulation indices for adult worm antigen-induced stimulation between the infected and control subjects, the levels being similar to those observed prior to infection.

**Serum antibodies.** Serum anti-hookworm antibodies of the IgM and IgG classes are shown in figure 5. IgM antibodies appeared in both infected subjects two weeks after infection and persisted for the duration of the experiment. Similarly, in one subject specific IgG antibodies appeared transiently two weeks after infection, declined by four weeks and rose again six weeks after infection and remained elevated for the remaining 24 weeks of infection. Levels of IgG antibodies in the second infected subject, however, did not appear until 12 weeks after infection and remained at a lower level compared with the other volunteer until the termination of the infection. Following treatment, levels of IgM and IgG antibodies declined in both subjects. Specific IgA anti-hookworm antibodies were not detected in either infected subject before or at any time after infection.

**Discussion**

The strain of *A. ceylanicum* that we used is capable clearly of producing patent infections in humans. One of the human volunteers (subject 1) had worked in hookworm-endemic areas for a short period of time more than 10 years prior to the infection described here but the other volunteer (subject 2) had not been exposed previously. Application of larvae to the skin was not followed by any reaction in either volunteer. This contrasts with the observations of Mapstone [4], Haydon and Bearup [5], and Wijers and Smit [6], all of whom reported a mild maculopapular 'ground-itch' in a few subjects. It is probably that the absence of reactions in our study was due to the considerable reduction in bacterial contamination of the infective larvae achieved by adding antibiotics to the PBS in which the inoculum was suspended.

There was little doubt that the sudden onset of severe abdominal pain and distention with excessive flatulence four weeks after infection was due to the ancylostome infection since both volunteers experienced a sudden onset almost simultaneously. Initially, the clinical upset was quite disabling. The first attack subsided over a week and was succeeded at irregular intervals by episodes of milder severity coincidently in both subjects. The clinical incubation period of four weeks was slightly longer than that noted by Wijers and Smit [6] who found that their subjects first developed abdominal distress between 15 and 20 days after infection. The symptoms that their volunteers experienced were very similar to those reported here and noted by Bearup [14] in his correspondence regarding the earlier report by Haydon and Bearup [5]. Indeed, we concur with the observation of Bearup [14] that the symptoms experienced appeared to be disproportionate to the few parasites present.

The onset of clinical manifestations preceded the appearance of eggs in the faeces by a few days. In both subjects, only small numbers of eggs were excreted in the faeces. We do not know the size of the intestinal worm burden but the low egg excretion would suggest that it was small. A previous report [7] found that less than 5% of the larvae given percutaneously to three volunteers matured. Egg excretion continued at the same levels for seven months until the infection was terminated with anthelmintics.
Blood eosinophilia is a well recognised concomitant of hookworm infection [15]. A significant elevation was seen about four weeks after infection and this persisted for between four and five weeks, then declined slowly. The course was similar to that seen by Bearup [14], except that the degree of eosinophilia was much less than he reported. Small levels of eosinophilia have been reported previously with naturally acquired A. ceylanicum infections. Anten and Zuidema [16] reported levels of 7% and 8% eosinophilia in two patients infected with this strain of hookworm.

No other studies have measured immunological responses in humans to A. ceylanicum infection. The most striking effect was the response of lymphocytes to stimulation with specific hookworm antigens. One infected volunteer showed a marked transitory rise in the larval-induced lymphocyte stimulation index, whereas the other infected subject failed to give any such reaction. This may result from possible sensitisation of the former volunteer to hookworm while being in the tropics. The time of maximum stimulation three weeks after infection follows the period of larval migration after which larvae presumably either mature or are destroyed or expelled from the gut. In contrast, lymphocytes from both infected volunteers demonstrated more sustained stimulation by adult worm antigen. This began about three weeks after infection and reached peak levels four and six weeks after infection, thus approximating the appearance of adult worms in the bowel. The more prolonged duration of lymphocyte responsiveness to adult worm antigen may reflect the persistence of worms in the gut. The lack of specific stimulation noted late in the course of infection may indicate modulation of this responsiveness, as has been noted in a number of other chronic parasitic infections [17]. Specific antibodies directed against both larval and adult worm antigens in experimental human hookworm infection have been measured previously but specific IgM and IgG antibodies were not delineated [18]. In this study the authors suggested that antibody response to infection may be important in limiting the severity of gastrointestinal disturbances. The small initial rise in IgG we observed in subject 1 may reflect possible previous exposure of this person to hookworm in Papua New Guinea and the Philippines. It is of interest that he also had the higher titres of IgG and IgM antibodies, the greater lymphocyte stimulation indices, and the more severe symptoms.

Thus, we have demonstrated that this strain of A. ceylanicum obtained originally from an infected dog in Malaysia is capable of complete development in man. Chronic low levels of infection persisted with this strain for 30 weeks prior to termination of the infection, in which time clinical symptoms, eosinophilia, lymphocyte blastogenesis and antibody responses were observed. This human parasite has been subsequently passaged back to dogs and a model of human hookworm infection established [12] which will enable host-parasite interaction to be analysed closely.

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References

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