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Immune responses following experimental human hookworm infection

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Summary

To characterize the immune response following primary human hookworm infection, an adult volunteer was infected with 50 L3 larvae of *Necator americanus*, reinfected 27 months later and followed for a further 6 months. Clinical signs, blood picture, *ex-vivo* peripheral blood cytokine production (IFN-γ, IL-5, IL-13, IL-10 to mitogen and hookworm antigen), acute phase proteins (APP) (C-reactive protein, CRP and α1-antitrypsin, α1-AT) and antibody levels were determined. Dermatitis, oedema, mild nausea and abdominal discomfort followed the primary infection. Eosinophil counts peaked early during both infections but remained elevated (~18%) throughout. Transient production of IL-5, IL-13 and APP also followed infection but there were negligible levels of IFN-γ or IL-10. The onset of nausea, oedema and the initial rise in CRP, α1-AT, eosinophilia and IL-5 coincided (days 13–27) with the late larval migration and early establishment of the preadult worms in the intestine. Apart from the eosinophilia these responses declined to baseline levels within 4 months and were less pronounced on re-infection.

Keywords: cytokines, eosinophils, hookworm, human, whole blood assay

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Introduction

In endemic countries infants are exposed to gastrointestinal nematode infections soon after birth and infection intensity gradually increases during childhood. Chronically infected humans from endemic areas show strong Th2 responses as judged by high levels of specific and nonspecific IgE and eosinophilia [1,2]. Such individuals show either a Th2-skewed cytokine response in the case of *Ascaris lumbricoides* infections [2] or a mixed Th1/Th2 response in *Trichuris trichiura* and hookworm infections [3–5]. Nothing is known about the cytokine response following primary exposure of humans to gut nematode infections but in mouse models initial infections in different inbred strains can induce polarized Th1 or Th2 responses, the latter correlating with rapid expulsion of the worms and Th1 responses with chronic infection [6]. Interest in the immunology of helminth infections has also increased in recent times because of their immunomodulatory effects which are known to involve TGF-β and IL-10 [7]. The primary purpose of this study was to characterize the cytokine and systemic acute inflammatory response in a human volunteer following primary and repeat infections with the hookworm *Necator americanus*.

Materials and methods

Subject

The volunteer was a male Caucasian, aged 51 at the study start, with no history of prior hookworm infection and a normal medical history. Ethical approval for the study has been granted by the ethics committee of the London School of Hygiene and Tropical Medicine.

Parasitology and infection

N. americanus eggs were purified using a saturated sodium chloride-flotation technique [8] from fresh stool recovered from infected subjects from Pemba Island, Zanzibar. Purified eggs were mixed with activated charcoal and fresh stool from the volunteer and cultured for 10 days at 26 °C using the Harada-Mori technique [9]. The larvae were stored at 4 °C. For the second infection, eggs were collected from the infected volunteer. For the infection 60 μl of a suspension of approximately 50 L3 larvae were pipetted onto a damp gauze pad placed onto the upper surface of the left forearm (or on the calf muscle for the 2nd infection) for 1 h. The second infection was 815 days after the first. The Formol-ether

concentration technique [10] was used to detect hookworm eggs and Charcot-Leyden crystals. The Kato-Katz technique [11] was carried out in duplicate to determine eggs per gram of faeces.

Haematology procedures

Capillary blood was used to make duplicate Giemsa stained thin blood films for differential blood cell determination and to determine the total white cell count using white cell dilution buffer and an Improved Neubauer haemocytometer.

Antigen preparation

Adult *N. americanus* maintained in a hamster life cycle (kindly provided by Prof J Behnke of Nottingham University) were homogenized in liquid nitrogen, the powder suspended in 1× PBS (Gibco, Paisley, UK), sonicated on ice and centrifuged at 11 160 ***g*** for 30 min at 4 °C. The supernatant was filter-sterilized, aliquoted and stored at −70 °C. Protein concentration was determined using the Bio-Rad protein assay. Phytohemagglutinin (PHA) was from Becton Dickinson (Oxford, UK).

Whole blood culture

These were carried out in duplicate. Heparinized blood (5 ml) was used within 1 h of venepuncture and diluted 1 : 5 with RPMI 1640 (Gibco) supplemented with 20 IU/ml penicillin (Gibco), 20 μg/ml streptomycin (Gibco), 2 mM L-glutamine (Gibco). Hookworm antigen was used at a final concentration of 30 μg/ml and mitogen at 5 μg/ml. 100 μl of each test antigen and appropriate controls were added to wells of 96 round-bottomed tissue culture plates (Nunc) to which 100 μl of diluted blood was added (giving a final blood dilution of 1 : 10) and incubated at 37 °C in an atmosphere of air and 5% CO_2. Supernatant was removed on day 6 of culture, pooled, realiquoted and stored at −20 °C.

Cytokine, antibody and acute phase protein ELISA

For the cytokine ELISA, matched monoclonal antibody pairs from Pharmingen (Oxford, UK) or R & D Systems (Abingdon, UK) were used according to the manufacturer's instructions (IL-5: TRFK5 and JES1–5A10, IL-10: JES3–9D7 and JES3–12G8, IL-13: JES10–5A2 and B69-2 from Pharmingen, IFN-γ from R & D Systems).

The antibody ELISAs were carried out as described elsewhere [12]. Hookworm antigen was used at 5 μg/ml for total IgG and 20 μg/ml for IgE. Test samples were diluted 1/100 for IgG and 1/20 for IgE in 1% BSA/PBST and alkaline phosphatase-conjugated detection antibodies were diluted 1/1000 for total IgG (Dako, Ely, UK) and 1/2000 for IgE (Becton Dickinson).

To measure CRP in human plasma, Nunc Immulon-2 plates were coated with sheep antihuman CRP (The Binding Site) (1/16 000 in 0·13 M borate pH 9·6). CRP standard (1 μg/ml −0·00195 μg/ml; Dade Behring, Milton Keynes, UK) or samples (1/25) were added with CRP-biotin conjugate (0·125 μg/ml, prepared using NHS-LC-Biotin (Pierce Endogen, Northumberland, UK) according to manufacturer's instructions) to all wells. For detection, streptavidin:alkaline phosphatase (1/2000, Serotec) was used with substrate solution (1 mg/ml p-nitrophenylphosphate (Sigma, Poole, UK) in 0·1 M $NaHCO_3$ pH 9·6, 2 mM $MgCl_2$). All incubations were for 1 h at room temperature and plates were washed in PBS/T and read at 405 nm. The same protocol was used for α1-antitrypsin measurement but using: rabbit antihuman α1-antitrypsin (1/4000, Dako), α1-antitrypsin standard (40 μg/ml −0·07812 μg/ml Protein-N-std; Dade Behring), α1-antitrypsin conjugate (1 μg/ml, biotinylation using biotin hydrazide; Sigma) and plasma samples were diluted 1/500.

Results

Clinical features

Within 16 min of applying the larvae, a stinging sensation was experienced leading to an itchy papular rash which persisted until day 47. Mild rawness was experienced in the lower respiratory tract between days 17–19. From day 26 there was occasional mild nausea and by day 45, moderate abdominal discomfort that could have been caused by an acute enteritis. Between days 27–42, the subject occasionally experienced oedema in both feet and on day 33 on the right arm. At the second infection the skin irritation was less and there were no respiratory or intestinal symptoms or oedema.

Patency

Eggs first appeared in the stool at day 53 post infection (p.i), peaked on day 145 p.i at 1176 epg and then remained around 600–900 epg. Following the second infection egg output increased markedly just 14 days post infection and reached a peak of 2604 epg at 128 days p.i (Fig. 1a). Charcot-Leyden crystals were noted in stool throughout the infections starting at day 42 p.i.

Blood picture

The total number of white blood cells (TBC) pre-infection was $6{\cdot}175 \times 10^9$ cells/l. This increased 2·5 fold to $15{\cdot}350 \times 10^9$ cells/l by day 42 p.i, declined over the next 28 days to $9{\cdot}350 \times 10^9$ cells/l and plateaued just above pre-infection levels. Re-infection promoted a marked but lower TBC increase (data not shown).

Eosinophil counts, with a baseline value of $0{\cdot}3 \times 10^9$cells/l (4·5% of TBC), increased between days 14–21 and reached $6{\cdot}370 \times 10^9$ cells/l (41·5% of TBC) on day 42 before declining to a persistently elevated level of around $1{\cdot}6 \times 10^9$ cells/l

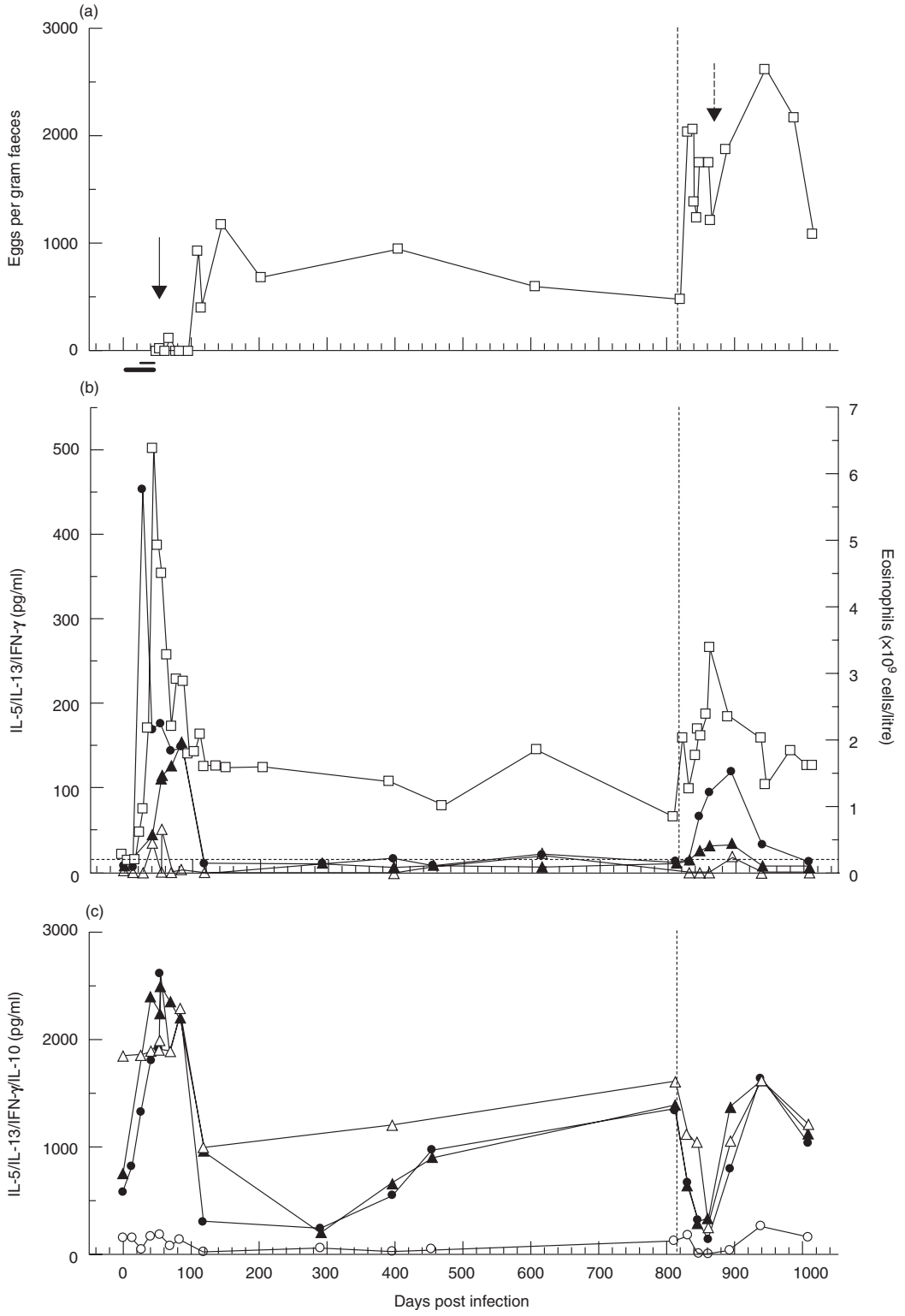

Fig. 1. (a) Faecal egg excretion during the course of infection. Dotted vertical line = second infection. Solid arrow indicates time of patency during the first infection and the dashed arrow indicates the same interval following the second infection. The short horizontal bars indicate the periods within which skin rash (thick bar) or peripheral oedema, nausea and abdominal discomfort occurred (thin bar). (b) Cytokine responses to somatic hookworm antigen in relation to blood eosinophilia during the course of infection. Left axis: cytokine concentration; right axis: eosinophil count. IL-5 (●), IL-13 (▲), IFN-γ (△), eosinophil count (□), dotted vertical line = second infection; dotted horizontal line = limit of cytokine detection. (c) Cytokine responses to PHA during the course of infection. IL-5 (●), IL-13 (▲), IFN-γ (△), IL-10 (○), dotted vertical line = second infection; dotted horizontal line = limit of cytokine detection.

(18% of TBC) (Fig. 1b). There was no such marked increase in absolute cell number for monocytes, lymphocytes or neutrophils (data not shown). During the second infection, there was a transient increase in eosinophilia, peaking at $3{\cdot}380 \times 10^9$ cells/l at 46 days p.i.

Cytokine responses to *N. americanus* antigen and PHA

Antigen specific IL-5 production increased markedly between days 13–27 post primary infection (Fig. 1b), and then dropped by day 41 reaching baseline values by 118 days. Levels rose again but less starting on day 15 after the second infection. Although the eosinophil peaks following both infections corresponded broadly with the peaks in IL-5, the elevated eosinophilia following the primary infection occurred in the absence of detectable IL-5 production by the WBA. IL-5 induced to an excretory-secretory (ES) hookworm antigen preparation followed a similar pattern although levels were lower (data not shown). An IL-13 response was slower to develop increasing at day 41 and reaching a peak at day 84 p.i before falling to baseline levels by day 118 (Fig. 1b). Following re-infection there was a slight increase between days 30 and 78 post infection falling again by day 122. Very little IFN-γ (Fig. 1b) and negligible levels of IL-10 (data not shown) were detected throughout the time course.

The response to PHA was also characterized by a marked but short-lived increase in IL-5 and IL-13 but not IL-10 or IFN-γ during the primary infection (Fig. 1c). Following re-infection, cytokine levels initially fell before recovering to pre-re-infection levels.

Hookworm specific IgG and IgE responses

Antigen specific IgG and IgE gradually increased during the primary infection (IgG: 53% increase from baseline levels to peak at day 614 pi; IgE: 715% increase by day 811) and were boosted following the secondary infection to a peak around 3 months later (IgG: 10% increase; IgE: 94% increase from pre-secondary infection levels) (data not shown).

Acute phase protein response: C-reactive protein and α1-antitrypsin

During the primary infection there was a very transient increase in CRP at days 20–34 (baseline 1·4 μg/ml to 8·1 μg/ml at day 34) (data not shown) but there was no increase during the second infection. Levels of α1-antitrypsin were elevated at days 20–118 after the initial infection (from baseline value of 1013·6 μg/ml to fluctuate around 1900 μg/ml) and around day 15 (from 519·5 μg/ml to 1156·7 μg/ml) following the second infection.

Discussion

Clinical symptoms were mild and relatively brief as in previous experimental infections [13–18] but the papular skin rash persisted longer (6 weeks) and oedema occurred during the primary infection and distal to the infection site. All the clinical symptoms were reduced or absent on re-infection.

Eggs first appeared in the stool at 53 days post exposure, consistent with other experimental infections [13–15,17] but did not show the rapid decline at 15–24 months previously reported [13]. Following the second infection there was a marked increase in egg count 14 days after infection, i.e. prior to the expected prepatent period for the incoming *N. americanus* infection based on the first infection and on previous reports (48–66 days) [13–15,17]. This may reflect direct effects of immunological factors on development or fecundity of the incoming or established adult worms similar to the direct effects of cytokines on *Schistosoma mansoni* [19,20].

Blood eosinophilia started to rise between days 14 and 21 post exposure and reached a peak around 42 days but unlike earlier studies when eosinophil levels fell to baseline following anthelminthic treatment [14,15,17,18], eosinophilia persisted for nearly two years and again showed a transient peak following re-infection. Thus although the tissue migrating stages may provide the major stimulus to eosinophilia [21], adult worms can maintain an elevated level. Charcot-Leyden crystals (CLC) appeared in the stool during the first peak in eosinophilia and persisted thereafter. CLC are associated with allergic inflammation [22] and specifically with eosinophils [23]. Since intestinal biopsies from humans with hookworm infection have demonstrated eosinophils in submucosal tissues [24] the presence of CLC in the gut probably reflects eosinophil recruitment to the infection site.

A previous study [5] of hookworm infected individuals from an endemic area showed elevated Th2 (IL-4 and IL-5) and Th1 (IFN-γ) cytokine production by purified PBMC to *N. americanus* ES antigen. However, following primary exposure we detected only transient Th2 cytokine production to both hookworm and PHA stimulation although not to the unrelated mycobacterial antigen, purified protein derivative (data not shown). We did not detect any elevated antigen or mitogen induced IFN-γ or IL-10. Certain helminth infections are associated with T cell hyporesponsiveness mediated by high levels of IL-10 and/or TGF-β [7] but there is limited evidence to date for systemic antigen specific induction of IL-10 in human hookworm infections even in endemic populations [3,12].

The transient nature of the IL-5 and IL-13 response in the WBA might suggest that there is either a brief antigenic stimulus or active suppression. However, the persistent eosinophil production indicates that there is ongoing production of IL-5 [25]. Possibly antigen from the migrating larval stages induces the initial systemic response whereas focal antigen presentation by the established adult worms in the mucosal associated lymphoid tissue can maintain local production of IL-5 by antigen specific T cells but these are not recruited to the systemic circulation. An alternative possibility is that

IL-5 is maintained by innate mechanisms which have been proposed to explain the elevated IL-5 levels seen as early as day 3 post infection with *N. americanus* in mice [26].

Regarding the parasite stage responsible for the various clinical and immunological effects seen following the initial infection there was coincidence between the onset of mild nausea and oedema (on day 26/27) and the initial rise in eosinophilia (days 14–21), CRP (day 20–27), α1-AT (day 8–20) and IL-5 to both antigen and PHA (day 13–27). Following re-infection the rise in eosinophilia, α1-AT and antigen specific IL-5 also occurred at a similar time. In *N. americanus* infections in neonatal hamsters peak numbers of L3 larvae reach the lungs on days 4–7 post infection and L4 larvae reach the intestine between days 8–14 moulting to the preadult stage around days 17–21 [27]. Whether the rate of development/migration is comparable in humans is uncertain but larvae have been recovered from sputum on day 12 post experimental infection [13]; clinical signs of respiratory infection have been observed on days 8–21 (mucosal erythema [14]) and days 5–15 (laryngeal irritation with cough [13]); and gastrointestinal signs (nausea and abdominal pain) start around 30 days post experimental infection ([14] and in the current study). So the rise in Th2 cytokines, APP and eosinophil responses could be initiated by the larvae migrating through the lungs or the establishment of the preadult worms in the intestine.

In conclusion, two light, persistent successive infections with *N. americanus* induced minimal long-term symptoms apart from elevated eosinophilia. A transient Th2 cytokine response was detected in the peripheral blood coincident with the late lung migration/early intestinal phase. The pathology, cytokine response, eosinophilia and acute phase protein responses were generally lower following re-infection perhaps reflecting the operation of local immunosuppressive mechanisms [7] but no IL-10 production was detected *ex vivo* in peripheral blood cultures.

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