DOSE-RANGING STUDY FOR TRIALS OF THERAPEUTIC INFECTION WITH *NECATOR AMERICANUS* IN HUMANS

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**Abstract.** Epidemiological studies suggest that a hookworm infection producing 50 eggs/gram of feces may protect against asthma. We conducted a dose-ranging study to identify the dose of hookworm larvae necessary to achieve 50 eggs/gram of feces for therapeutic trials of asthma. Ten healthy subjects without asthma or airway hyperresponsiveness to inhaled methacholine received 10, 25, 50, or 100 *Necator americanus* larvae administered double blind to an area of skin on the arm. Subjects were seen weekly for 12 weeks and were then treated with mebendazole. Skin itching at the entry site and gastrointestinal symptoms were common at higher doses. Lung function did not change. Levels of blood eosinophils and IgE increased transiently, and levels of IgG increased progressively. All doses resulted in at least 50 eggs/gram of feces in the eight subjects who completed the study. Infection with 10 *N. americanus* larvae is well tolerated, elicits a modest host eosinophil response, and is potentially suitable for use in preliminary clinical therapeutic trials.

**INTRODUCTION**

Hookworms are successful parasites that currently infect approximately 740 million people worldwide. At high infection intensity, they cause significant morbidity and mortality. However, it has also been suggested that infection with hookworms or other intestinal parasites may have some beneficial effects in human health, and in particular protect against asthma and allergy, and that atopc persons may be indeed protected against hookworm infection. It is now well established that hookworms are able to survive and reproduce in the host as a consequence of complex immunomodulatory mechanisms involving a variety of immunoregulatory molecules such as interleukin-10 (IL-10), transforming growth factor-β (TGF-β), and metalloproteases. Since all of these mechanisms are also likely to inhibit the IgE-mediated allergic components of asthma pathogenesis, it is plausible that parasite infection might also protect against asthma. New evidence in support of this hypothesis has recently been presented in a range of epidemiological and animal studies.

Hookworm infection may therefore have therapeutic potential in the treatment or prevention of asthma, but the dose of hookworm larvae necessary to establish a protective and non-pathogenic infection intensity for experimental studies is unknown. Our epidemiological findings suggest that protection against asthma may be related to the intensity of parasite infection, being most evident in infections generating more than 50 eggs/gram of feces. If so, then experimental studies of therapeutic effects of hookworm infection in asthma should aim to establish infection at this level while minimizing symptoms and other adverse effects. We therefore report a dose-ranging study in healthy volunteers to identify the dose of hookworm larvae necessary to achieve these objectives.

**METHODS**

Study participants were recruited by local advertisement and word of mouth after initial screening to exclude those with a history of asthma, anemia, or any other significant medical disorder, or who were pregnant or of child-bearing potential and not willing to use an effective means of contraception for the duration of the study. Eligible subjects attended a baseline visit at which written informed consent was obtained and blood taken for hemoglobin estimation, differential cell counting (full blood count), and hookworm sero logic analysis. Lung function was measured using a MicroSpiro spirometer (Micro Medical Ltd., Kent, United Kingdom) by taking the one-second forced expiratory volume (FEV1) as the higher of two values within 100 mL, and the higher of two measures of forced vital capacity (FVC). Airway responsiveness to methacholine was measured by the method of Yan and others to a maximum cumulative dose of 24.5 μM, stopping the test if FEV1 decreased by ≥20% from the post-saline baseline value. Subjects with a ≥20% decrease in FEV1 were excluded from further participation. Allergen skin sensitization to *Dermatophagoides pteronyssinus*, cat fur, grass pollen, and histamine and saline controls were measured by standard skin prick test methods that measured the response at 15 minutes as the mean of two diameters at right angles to each other, one of which was the largest measurable diameter.

Consenting subjects were then seen on a separate occasion when a drop of water containing either 10, 25, 50, or 100 *Necator americanus* larvae was administered double-blind under gauze to an area of skin on the non-dominant arm. Randomization was in blocks of four according to a computer generated random code. *Necator americanus* larvae were obtained by culture of fecal material from a healthy human source known to be negative for human immunodeficiency virus and hepatitis B and C, as described by Kumar and Pritchard and stored in water until used.

After infection, subjects were seen weekly for up to 12 weeks. On each occasion, we measured FEV1 and FVC, obtained blood for a full blood count and immunoglobulin estimation, and collected a stool sample to quantify egg production. Stool egg counts were carried out by suspending a weighed sample of feces in 1–2 mL of saturated salt solution, counting eggs in a MacMaster egg counting chamber under a pre-marked grid, and back-calculating to estimate eggs per gram of feces. Between visits, subjects completed daily diaries of adverse effects, scoring severity on an arbitrary 0 (no symp-
toms) to 10 (maximum possible severity of symptoms) scale. At 12 weeks, allergen skin tests were repeated together with the other study measures, and subjects treated with 100 mg of mebendazole twice a day for three days. An additional fecal sample was checked two weeks later to ensure the infection was eradicated. Total IgE and specific IgG levels were estimated by standard enzyme-linked immunosorbent assays (ELISAs), and antigen recognition by post-infection sera was analyzed by Western blot, the methods for which are described below.

**Detection of total IgE.** Ninety-six well polystyrene plates were coated overnight at 4°C with mouse anti-human IgE (50 μL, 5 μg/mL) diluted in 0.05 M carbonate/bicarbonate buffer, pH 9.6, clone G7-18; BD PharMingen, San Diego, CA). The plates were washed with phosphate-buffered saline (PBS)/Tween 20 (0.05% v/v), pH 7.2 (PBS/Tween) and blocked with bovine serum albumin (200 μL of a 1% w/v solution) in PBS (BSA/PBS) for one hour at room temperature. After blocking, the plates were washed and 50 μL of serum (diluted 1:20 in 1% BSA/PBS) added to each well and incubated overnight at 4°C. In addition, 50 μL of human IgE standards (doubling dilutions from 100 IU/mL to 1.56 IU/mL) were included on each plate. All assays were carried out in duplicate. After overnight incubation, the plates were washed and 50 μL of biotinylated mouse anti-human IgE (2 μg/mL) diluted in PBS (diluted 1:1,000 in PBS) (clone G7-26; BD PharMingen) added to each well and incubated at for two hours at room temperature. The plates were then washed and 50 μL of streptavidin conjugated to horseradish peroxidase (diluted 1:1,000 in 1% BSA/PBS) was added to each well and incubated for one hour at room temperature. The plates were washed and developed with 100 μL of 3, 3′, 5′-tetramethylbenzidine (TMB) containing 2 μL of hydrogen peroxide per 10 mL of TMB. The reaction was stopped by adding 20 μL of 2.5 M sulfuric acid and the absorbance of each well measured at 450 nm.

**Detection of specific IgG.** Fifty microliters of *N. americanus* excretory-secretory (ES) products (5 μg/mL in 0.05 M sodium carbonate/bicarbonate buffer, pH 9.6) were used to coat the wells of a 96-well polystyrene plate overnight at 4°C. The plate was washed with PBS/Tween and the wells were blocked with 200 μL of 5% skimmed milk powder/PBS (blocking agent) for one hour at 37°C. The plate was washed as before and 50 μL of serum (diluted 1:500 in skimmed milk powder/PBS) added to individual wells and the plate was incubated overnight at 4°C. In addition, standards of known anti-hookworm IgG concentration (doubling dilutions from 2.8 μg/mL to 0.022 μg/mL) were also included on the plate. The plate was washed and 50 μL of sheep anti-human IgG conjugated to horseradish peroxidase (Binding Site, San Diego, CA) diluted 1:1,000 in blocking agent was added to individual wells and the plate was incubated at room temperature. The plates were washed and antibody binding was visualized by the addition of 100 μL TMB prepared as described above. The reaction was stopped by adding 20 μL of 2.5 M sulfuric acid and the absorbance of each well measured at 450 nm. All assays were carried out in duplicate. The ELISA values are expressed as the absorbance at 450 nm after the subtraction of a negative control value.

**Western blotting.** *Necator americanus* ES products (10 μg/lane) were separated under reducing conditions by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Western blots were blocked for one hour in 5% (w/v) skimmed milk powder in TBS at room temperature. Naive and post-infection serum (diluted 1:200 in 5% [w/v] skimmed milk powder/TBS) was added to the individual strips and incubated overnight at 4°C. Blots were washed with TBS/0.05% (v/v) Tween 20 and then incubated in sheep anti-human IgG (Binding Site) diluted 1:1,000 in 5% (w/v) skimmed milk powder/TBS for two hours at room temperature. The blots were then washed and developed in 4-chloro-1-naphthol (1 mg/mL) containing 0.02% hydrogen peroxide. Data on adverse effects and stool egg counts were monitored as the study progressed by the trial statistician who was blinded to allocation. We initially intended to randomize five volunteers to each of the four doses. The study was reviewed and approved by the Nottingham National Health Service and University ethics committees. All data were entered and analyzed using simple parametric methods as appropriate in SPSS version 13 (SPSS Inc., Chicago, IL).

**RESULTS**

Ten people (five men) 24–57 years of age participated in the study. One participant in the first block of four to be randomized developed an extensive maculopapular eruption at the larval entry site within one day of infection, and at five weeks experienced recurrent vomiting and diarrhea. She was withdrawn as a result of the gastrointestinal disturbance and treated with mebendazole. Her treatment code was broken and showed that she had received 100 larvae. No further allocations were made to that dose. Three participants were randomized to each of the lower doses, one of whom also developed diarrhea and abdominal pain and withdrew and was treated at four weeks; breaking the code for this individual established that he had received 50 larvae. The other eight participants went on to complete the full 12-week study and attended a minimum of 10 of the 12 weekly visits. Recruitment was stopped after three subjects had been allocated to each dose because it became apparent from monitoring the fecal egg count data that the objectives of the study had been met (Figure 1).

**Symptoms.** *Local skin reactions.* Nine participants reported immediate local skin itching and developed a localized maculopapular rash at the skin entry site that typically lasted for 2–5 days (Figure 2). The person who received 100 larvae had a severe eruption that lasted 21 days. The participant who received 10 larvae developed diarrhea and abdominal pain and withdrew and was treated at four weeks; breaking the code for this individual established that he had received 50 larvae. The other eight participants went on to complete the full 12-week study and attended a minimum of 10 of the 12 weekly visits. Recruitment was stopped after three subjects had been allocated to each dose because it became apparent from monitoring the fecal egg count data that the objectives of the study had been met (Figure 1).

**Figure 1.** Study design and allocation of hookworm larval doses.
Gastrointestinal symptoms. Abdominal discomfort was reported by nine participants and tended to be intermittent and in some cases related to meals. Most participants also reported occasional episodes of diarrhea, and in some cases nausea. Two reported early satiety, and six increased flatulence. At the lower doses, abdominal discomfort did not occur until at least three weeks after infection (Figure 3).

Respiratory symptoms. One participant who received 10 larvae reported cough productive of a small amount of phlegm during week seven, and one who received 25 larvae reported mild wheeze (score 2/10) on the second day of infection. The subject who received 50 larvae and withdrew after four weeks reported cough on days 5, 15, and 16-post infection and mild wheeze (score 3/10) during the third week. The subject who received 100 larvae reported mild breathlessness (score 2/10) on day 11.

Malaise, fatigue, and other symptoms. Symptoms of malaise or fatigue were reported by four subjects. Symptoms were mild to moderate (maximum score 5/10) and occurred between weeks six and seven in three subjects and at week 12 in one subject. Other symptoms reported by individual subjects were mild neck and headache for two days by one subject after a period of working at a computer and pyrexia (38°C) and coryzal symptoms for two days during week seven by one subject.

Response of symptoms to therapy. All symptoms settled completely after subjects were treated with mebendazole.

Lung function and allergen skin tests. Mean (SD) FEV$_1$ and FVC at baseline were 3.6 (0.5) and 4.5 (0.9) liters and at exit from the study were 3.6 (0.5) and 4.3 (0.8), respectively. Overall, these did not change significantly on average or in any individual during the study ($P = 0.52$ and $P = 0.09$ for change in FEV1 and FVC, respectively, by paired t-test).

Only one participant who completed the study had positive allergen skin test results. Saline-adjusted wheal diameters in this individual at baseline were 5 mm to cat fur and 7 mm to grass pollen, these decreased to 4 mm and 5 mm, respectively, at the end of the study.

Leukocyte counts and hemoglobin levels. The mean white blood cell count increased to a peak at 5–9 weeks post-infection (Figure 4) almost entirely because of changes in eosinophils (Figure 5). All other leukocyte counts remained within normal ranges throughout the study. The increase in eosinophil count was lower in the 10 larvae group (Figure 5). Hemoglobin levels remained stable throughout the study.

Egg counts. Hookworm eggs were not seen in fecal samples at any time in the two subjects who withdrew from the study prematurely, but appeared at between four and six weeks after infection in all participants who completed the study. The highest egg counts occurred in the people who received 50 larvae; median egg counts were similar in participants allocated to the two lower doses (Figure 6). Egg counts for individual subjects were variable and two subjects had one or two weeks in which eggs were not detected. All participants had negative egg counts two weeks after treatment with mebendazole.

Immunoglobulins. Total IgE levels increased slightly during weeks 2–6 in the two higher dose groups, but overall there was little difference between treatment groups (Figure 7). Specific IgG levels increased gradually from time of infection.
peaking at week 10 in those subjects who received 10 larvae and at 12 weeks in the 2 higher larval dose groups (Figure 8). Western blots of adult hookworm secretions were probed with baseline sera and sera taken from the final bleeding prior to treatment. Eight of 10 volunteers had a typical IgG antigen recognition profile to hookworm infection. The two volunteers who terminated the study early failed to show a specific IgG response to hookworm secretions.

**DISCUSSION**

This study has explored the dose-related effects of hookworm infection in healthy volunteers and demonstrates that infection with cutaneous doses of ≥ 10 larvae generated an intensity of infection resulting in fecal egg counts > 50 eggs per gram, which our previous work suggests may be the approximate threshold of intensity necessary to offer protection against asthma and allergic disease. A range of predominantly dose-related adverse effects was reported.

The initial adverse effect in most participants was a localized pruritic skin reaction at the site of larval entry, which began within a day or so of infection and typically lasted for up to a week. In approximately half of the participants the rash relapsed or recurred approximately 2–3 weeks after infection for up to 10 days before disappearing. This biphasic effect probably reflects the lag period required for the immune system to mount a response to larval antigens deposited in the skin soon after entry. However, the most troublesome...
adverse effects were gastrointestinal symptoms, and these were the cause of the two withdrawals from the protocol. The gastrointestinal disturbances appeared to be dose-related; however, they were mild and well tolerated among the participants who received 10 larvae. The other most commonly reported adverse effect was malaise or fatigue, occurring between weeks six and seven after infection. These symptoms occurred when eosinophil counts were at their highest and may have been indirect effects arising from systemic eosinophilia rather than directly from the parasite.

A major concern in exploring the therapeutic potential of hookworm infection in asthma, and in other conditions, is the theoretical risk of pulmonary adverse effects during the lung migration phase. For this reason we excluded all subjects with a history of asthma or with measurable airway hyperresponsiveness from the present study, and monitored FEV, vital capacity, and respiratory symptoms throughout the study. We found no evidence of change in lung function at any time, but symptoms of cough, breathlessness, or wheeze were reported by three participants during the first four weeks of infection, the period during which lung migration occurs. However, the cough was troublesome only in one subject and for one day, while the reported wheeze and breathlessness were not prominent. None of the three subjects who received 10 larvae reported any respiratory symptoms. Our findings on adverse effects thus demonstrate that of the four doses tested, the 10 larvae dose was the best tolerated. However, since the total IgE and eosinophil responses to this dose were also less than with the higher doses, it is likely that this greater tolerability is achieved at the expense of a lesser effect in provoking a potentially beneficial (anti-asthma) host immune response.

Iron deficiency anemia resulting from chronic gastrointestinal blood loss is the most important complication of hookworm infection in parasite-endemic tropical areas where infected individuals can carry high loads of worms and are subject to repeated infections over their lifetime. Anemia was not apparent in our study, and given the short duration of infection and the fact that our subjects were all well nourished, was unlikely to pose a problem.

Infection with the higher doses also provoked a substantial increase in the number of circulating eosinophils (Figure 5). Levels of specific IgG increased in all groups throughout the trial period (Figure 8), while total IgE responses to all doses were relatively low and failed to show any significant differences between infection groups (Figure 7). Specific IgG responses on Western blots demonstrated a typical antigen recognition profile for infected humans at all doses of hookworm larvae (Figure 9). However, this response was not found in post-infection serum samples of the two subjects (4 and 12) who terminated the study early. This implies that infection beyond five weeks is required to detect this immune response and may be linked to L4 larval entry into the gastrointestinal tract, which occurs around this time, and this event has been associated in the past with the onset of eosinophilia. Further analysis of the immunologic responses to infection, including IL-2, IL-4, IL-5, IL-10, TGF-β, interferon-γ, and basophil

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**Figure 6.** Median number of hookworm eggs per gram of feces in the eight subjects who completed the study, by dose of hookworm larvae.

**Figure 7.** Geometric mean IgE level in the eight subjects who completed the study, by dose of hookworm larvae.

**Figure 8.** Geometric mean specific IgG, by dose of hookworm larvae.

**Figure 9.** Western blotting of specific IgG in naive and 12-week post-infection serum samples.
challenges with IgE and hookworm allergens, is in progress and will be reported elsewhere.

Previous studies have also documented the effect of experimental hookworm infection in humans over the last three decades but in small numbers of subjects. Turton and Ogilvie and others reported self infection with 250 N. americanus larvae, which established infection at an intensity of > 3,500 eggs per gram of feces. However, as in our study, this infection did not have a marked effect on total IgE level. In another study, the larvicial effects of albenza-zole were compared with placebo in 29 volunteers exposed to 45 larvae. The placebo group had a mean egg count of 268 per gram of feces, adverse effects, and an eosinophil response broadly similar to those in participants receiving 50 larvae in our study. In a further study in which five volunteers were followed-up for up to 10 weeks after dosing with 50 larvae, the incidence of adverse effects, eosinophilia, and total IgE responses were also broadly similar to our findings; one of the five participants studied also had to withdraw because of gastrointestinal disturbance. Beaver followed-up an individual infected with three larvae whose initial egg counts of 1,000 per gram of feces decreased to zero over the course of 18 years. This concurs with the results of the study of Palmer, which investigated periodic egg counts from an individual given an undetermined number of cultured N. americanus larvae. Egg production leveled off at 11 months and remained constant for 6 years, thereafter decreasing until egg production ceased at 15 years.

One obvious source of bias in the present study was the inclusion of four of the authors as participants. Although not conventional, we believed that on moral and ethical grounds we could not recruit volunteers to the study without being willing to undergo infection ourselves. The doses received by the authors, as for all participants, were double-blind and allocated at random, but at the end of the study proved to be 25 in one instance and 50 in the other three instances. The low incidence of adverse effects in the 10 larvae group in particular is therefore not attributable to bias arising from possible underreporting of adverse effects by participants who were also investigators.

The use of parasites in this manner is not restricted to type I hypersensitivity reactions. Two recent studies have examined therapeutic use of another nematode, Trichuris suis, in inflammatory bowel disease, which is also an immune-mediated condition. In both active Crohn’s disease and ulcerative colitis, Trichuris was well tolerated and appeared efficacious in treatment of symptoms.

We conclude that infection with low doses of N. americanus, particularly with 10 larvae, is well tolerated, and elicits a modest host eosinophilic and antibody response. It is therefore potentially suitable for use in preliminary clinical therapeutic trials looking at its effects on modulation of the allergic response in atopic individuals and we wait with interest to see the results of such studies.

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Disclosure: D. Pritchard wishes to disclose that he is an inventor on a patent supporting the use of molecules derived from nematodes as immune modulatory agents. The study could be viewed as supportive of such intellectual property if any ensuing trial is successful. This statement is made in the interest of full disclosure and not because the author considers this to be a conflict of interest.

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