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THE CLINICAL AND IMMUNOLOGIC RESPONSES OF NORMAL HUMAN VOLUNTEERS TO LOW DOSE HOOKWORM (*NECATOR AMERICANUS*) INFECTION

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Abstract. Five normal human volunteers were exposed to approximately 50 infective larvae of *Necator americanus* and were observed for the development of clinical signs or symptoms and for changes in blood eosinophil levels, IgG antibody titers, total and parasite-specific IgE, and lymphocyte blastogenic responses for 6–10 weeks. Bronchoalveolar lavage was performed on four subjects prior to infection and at times when larval migration through the pulmonary tree was likely.

Eggs were demonstrated in the stools of four volunteers who remained untreated for more than 6 weeks; one volunteer had to be treated at day 40 because of severe gastrointestinal symptoms. All others also complained of abdominal pain and flatulence between days 35–40.

All volunteers developed marked blood eosinophilia which peaked between days 38–64 and ranged from 1,350–3,828 eosinophils/mm^3. Small increases in total and parasite-specific IgE and IgG were noted in some volunteers. One volunteer showed a significant lymphocyte blastogenic response. With the exception of mucosal erythema, bronchoalveolar lavage results were unremarkable.

Our data indicate that a single small inoculum of hookworm larvae is capable of producing significant transient gastrointestinal morbidity and marked blood eosinophilia but does not induce other prominent T cell- and B cell-dependent immune responses.

Ancylostoma duodenale and *Necator americanus* are the two hookworm species most commonly infecting humans.[1, 2] The most serious clinical manifestation associated with this infection is iron deficiency anemia caused by direct blood loss into the gastrointestinal tract at the points of parasite attachment.[1–3] Such blood loss becomes clinically significant when infection intensity is great and dietary intake of iron is poor;[3] most infected individuals with lesser parasite burdens are asymptomatic. Between these two extremes are patients with a variety of clinical manifestations whose determinants are poorly understood but which likely relate to differences in levels of infection intensity and/or immunologic sensitization. These include the cutaneous manifestations of pruritis and dermatitis commonly known as "ground itch" that develop following penetration of the infective larvae through the skin; pulmonary manifestations such as cough, wheezing, bronchitis, or pneumonitis that are uncommon but occur in relation to larval migration through the pulmonary tree to the gastrointestinal tract; and gastrointestinal disturbances which can include nausea, vomiting, diarrhea, and abdominal pain, often with postprandial accentuation.[1–5]

Few studies have addressed the immunologic determinants or correlates of hookworm disease.[6–10] In chronically infected populations patients usually show blood eosinophilia and have both elevated total serum IgE levels and specific antibodies that can be detected by fluorescence, complement fixation, ELISA, and hemagglutination.[6–8, 10] The clinical implications of these immunologic findings, however, are uncertain because in such naturally infected populations there are many variables (e.g., duration of infection, number of infecting organisms, and coexistence of other parasitic infections) that remain entirely uncontrolled.

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Earlier studies of experimental infections have indicated that the hookworm antibodies appear as early as 6 weeks post-infection (ELISA) but are incapable of conferring resistance to reinfections[6-8, 10] and that lymphocyte blastogenic responses to hookworm antigen can be demonstrated by day 17 of a primary infection.[9]

In order to reexamine these immunologic findings and to evaluate the potential of experimental infection as a model to study the development of immune responses to invasive helminth infections in humans, we undertook a comprehensive evaluation of five normal volunteers experimentally infected with *Necator americanus*. Each was exposed to approximately 50 infective third stage larvae of this parasite, and clinical and immunologic responses were followed over an 8–10-week period.

MATERIALS AND METHODS

Volunteers

Five male volunteers were recruited from the normal volunteer office of the National Institutes of Health. Prior to entry into the study, all had normal medical history, physical exam, complete blood count and differential (CBC), urinalysis, stool examination for parasites, chest x-ray, electrocardiogram, blood chemistries, pulmonary function tests, and sputum cultures. None had a history of prior hookworm infection. The volunteers ranged in age from 21–40 years, in height from 5′10″–6′1″, and in weight from 150–180 pounds. Four were white, one was black; all were born in the U.S. All studies were carried out under protocols approved by the National Institutes of Health, and in all cases informed consent was obtained.

Infection, clinical monitoring, and special procedures

The five volunteers were exposed to approximately 50 actively motile larvae of *N. americanus* from a known source following a method previously described.[5] In brief, a suspension of 50 larvae was pipetted onto a gauze pad placed on the volar surface of the forearm for 30 min. The forearm was examined for penetration sites and photographed. The volunteers were followed clinically and their blood sampled at weekly or biweekly intervals for CBC, serum chemistries, and immunologic evaluation. Cellular immune studies were performed immediately, but all sera were stored at −70°C until analyzed. Stools were examined for eggs by the Beaver standard smear technique.[11]

Skin biopsies of the penetration sites were performed using a 4-mm Baker's biopsy punch (Baker-Cummins, Key Pharmaceuticals, Miami, Florida). Bronchoalveolar lavage was performed on four of the five volunteers using a fiberoptic bronchoscope (Olympus BF Type 10) as previously described.[12] In brief, single subsegments of the lingula, right middle and left lower lobes were lavaged with five 20-ml aliquots of sterile saline by sequential instillation and aspiration for a total of 100 ml instilled into each lobe. Differential cell counts were made on cytocentrifuge preparations.

The infections were terminated with mebendazole (100 mg b.i.d. × 2 days) after 60 days in all but one volunteer who required treatment at day 40. Follow-up stool examinations on all volunteers were negative for hookworm eggs.

Antigen preparation

Soluble extracts of third stage larvae of *N. americanus* were used as sources of antigen. A Branson (sonicator) Model 575 with microprobe at a power setting of #6 was used (manufactured by Heat Systems, Long Island, New York). About 1,200,000 third stage larvae were washed with phosphate buffered saline (PBS) pH 7.4, and sonicated for 2 min. The sonicate was extracted with 8 ml of PBS at 4°C with gentle stirring for 12 hr, after which the suspension was centrifuged at 10,000 rpm for 30 min. The antigens were assayed for protein by the method of Lowry et al.[13]

IgG antibody determination

Parasite-specific IgG was measured by enzyme-linked immunosorbent assay (ELISA). Flat-bottomed 96-well microtiter plates (Dynatech Laboratories, Alexandria, Virginia) were coated with 0.1 ml carbonate bicarbonate buffer, pH 9.6, containing 2 μg/ml *N. americanus* antigen and allowed to incubate overnight at 4°C. The plates were washed in PBS containing 0.05% Tween 20. Samples were appropriately diluted and added to wells in 0.1 ml volumes and incubated at 37°C for 2 hr. The plates were then washed as before and 0.1 ml of a 1:500 dilution

of heavy chain-specific goat anti-human IgG conjugated to alkaline phosphatase was added to each well. The plates were incubated at 37°C for 2 hr, washed again and allowed to react with P-nitrophenyl phosphate disodium (Sigma, St. Louis, Missouri) in a sodium carbonate buffer at pH 8.6 and read after 30 min in an ELISA reader (Dynatech MR 600). A high titered serum from a Southeast Asian refugee with heavy hookworm infection was assigned arbitrary units. The calibration curve generated with this serum was then used to calculate antibody units in all test sera and the results were expressed as U/ml of neat serum.

IgE immunoglobulin and antibody determination

Total serum IgE was measured by radioimmunoassay with Phadebas IgE Prist kits (Pharmacia Diagnostics, Uppsala, Sweden). Parasite-specific IgE was determined using a solid phase radioimmunoassay. Flat-bottomed "remove-a-well" microtiter plates (Dynatech Immulon 1) were coated with 2 μg/ml of soluble *N. americanus* third stage larval antigen as described above. The wells were washed as described for the IgG antibody assay. Appropriate dilutions of the same reference serum used as the IgG standard and with proven high titer IgE antibodies and of test sera were incubated with the antigen overnight at 4°C. The plates were washed and subsequently incubated with ^{125}I-radiolabeled anti-IgE.[14] The second incubation was carried out for 4 hr at room temperature. The wells were washed, removed, and counted in a gamma counter. A similar plate coated with anti-IgE was incubated with a standard serum with known amounts of IgE and was run in the same assay simultaneously. The method of heterologous interpolation was used to approximate the amount of parasite-specific IgE bound to the plates.[15] Briefly, the assumption was made that the IgE detected by the ^{125}I-labeled anti-IgE reflected proportionately the amount of IgE bound to either the antigen plate or the anti-IgE plate since the identical probe (^{125}I-anti-IgE) was used in both cases. The calibration curve obtained with known amounts of IgE on the anti-IgE plate was then used as standard for interpolating the amount of antigen-specific IgE in the high titered serum. This high titered serum was used as the standard reference with a defined amount of parasite-specific IgE, and the values of all test sera were determined in comparison to it.

Lymphocyte blastogenesis

Mononuclear cells were obtained from heparinized venous blood by sedimentation over hypaque-ficoll in a standard fashion.[17] Cells were washed three times with RPMI 1640, supplemented with HEPES, glutamine, and gentamicin as previously described,[16] and 10^5 cells were cultured in the presence of either autologous or normal human AB sera (10%) in round-bottomed 96-well microtiter plates. Varying concentrations of phytohemagglutinin (PHA; Wellcome), concanavalin A (Con A; Pharmacia), tetanus toxoid (Massachusetts Public Health Laboratories), and soluble antigens of *Necator* were added to the cell cultures which were incubated at 37°C for 3 days (mitogens) or 6 days (antigens). After a 16-hr pulse with tritiated thymidine (New England Nuclear, Boston, Massachusetts) the cells were collected on glass filters with a PHD cell harvester (Cambridge Technologies, Cambridge, Massachusetts). Incorporated radiolabeled thymidine was measured by a liquid scintillation counter. Results are expressed as stimulation ratios (E/C).[16]

RESULTS

Clinical response

Cutaneous and gastrointestinal symptoms were seen after infection (Table 1). All five volunteers developed papular erythematous rash at larval penetration sites which were counted to estimate the number of larvae entering the skin. As described in a previous report,[5] it was easier to count the sites after about 48 hr had elapsed. We estimate that 35–40 larvae penetrated each volunteer; very few larvae were recovered after removal of the gauze pad. The rash was intensely pruritic early, diminishing generally within 72 hr. In two of the five volunteers, skin biopsies of the penetration sites were performed at 5 days post-infection. One showed a moderate perivascular chronic inflammatory infiltrate consisting primarily of lymphocytes but with occasional eosinophils; the other was unremarkable.

No volunteer had gastrointestinal symptoms until day 30. However, between days 30–45 all reported flatulence and epigastric/abdominal pain

TABLE 1

Clinical findings in five experimental Necator americanus *infections*

Symptoms/findings	Day of infection			
	1–6	30–45	48–50	113
Pruritus	(5/5) 100%			
Rash	(5/5) 100%			
Nausea		(2/5) 60%		
Vomiting		(1/5) 20%		
Diarrhea		(1/5) 20%		
Abdominal pain/discomfort*		(5/5) 100%		
Increased flatus		(5/5) 100%		
First appearance of hookworm eggs in stool			(3/4†) 75%	(1/4) 25%‡

* Two noted postprandial accentuation.
† One volunteer treated with mebendazole at day 40.
‡ No stools examined between days 45 and 113.

or discomfort (two with postprandial accentuation). Some complained of nausea (2/5), diarrhea (1/5), and vomiting (1/5). Three volunteers described the symptoms as mild to moderate; one reported them moderate to severe; and in one the symptoms were so severe that treatment was necessary at day 40 after infection.

Fecal samples from three of the volunteers were positive for hookworm eggs between days 48–58 (500–1,200 eggs/g of stool). One volunteer was temporarily lost to follow-up after day 45 but was positive on day 113 (200 eggs/g). We never were able to demonstrate eggs in the stool of the volunteer treated at day 40.

An evaluation for pulmonary signs of infection was done using bronchoalveolar lavage in four volunteers between days 8–21 of infection, the time when larval migration through the pulmonary tree should be taking place. All subjects had erythema of the bronchial mucosa during this period, and one had eosinophils comprising 2% of the recovered lavage cells (Table 2). Otherwise none of the findings from these studies differed from normal or preinfection values.

Immunologic response

Blood eosinophil counts increased progressively after 2–3 weeks in all volunteers, peaking between days 38–64 at levels that ranged from 1,350 to 3,828 cells/mm^3 (Fig. 1). A rise in the total white blood cell count mirrored this developing eosinophilia (Fig. 2); other blood leukocytes showed no significant changes. A spontaneous decline of the eosinophil number occurred in three volunteers, and after treatment with mebendazole all showed a fall in eosinophilia toward preinfection levels.

Lymphocyte transformation tests were carried out in four volunteers to evaluate responses to mitogens (PHA, Con A) and antigens (hookworm and tetanus toxoid) before infection and weekly (in one volunteer) or every 2–3 weeks during infection (three volunteers). All volun-

TABLE 2

Bronchoalveolar lavage data from four volunteers infected with Necator americanus

Volunteer	Day of infection	Total cells	Lavage cell differential*				Appearance of mucosa
			M	L	N	E*	
1	Preinfection	84 × 10^6	89	10	1	0	Normal pink
	8	78 × 10^6	91	7	2	0	Erythematous, friable
	22	67 × 10^6	86	11	3	0	Slight erythema, no friability
2	15	29 × 10^6	91	6	1	2	Mild erythema
3	21	27 × 10^6	68	32	0	0	Mild erythema
4	Preinfection	21 × 10^6	79	18	2	1	Normal pink
	8	31 × 10^6	97	3	0	0	Mild erythema
Normal values†		15–30 × 10^6	93 ± 3	7 ± 1	<1	<1	Normal pink

* M = macrophage; L = lymphocyte; N = neutrophil; E = eosinophil.
† See reference 12.

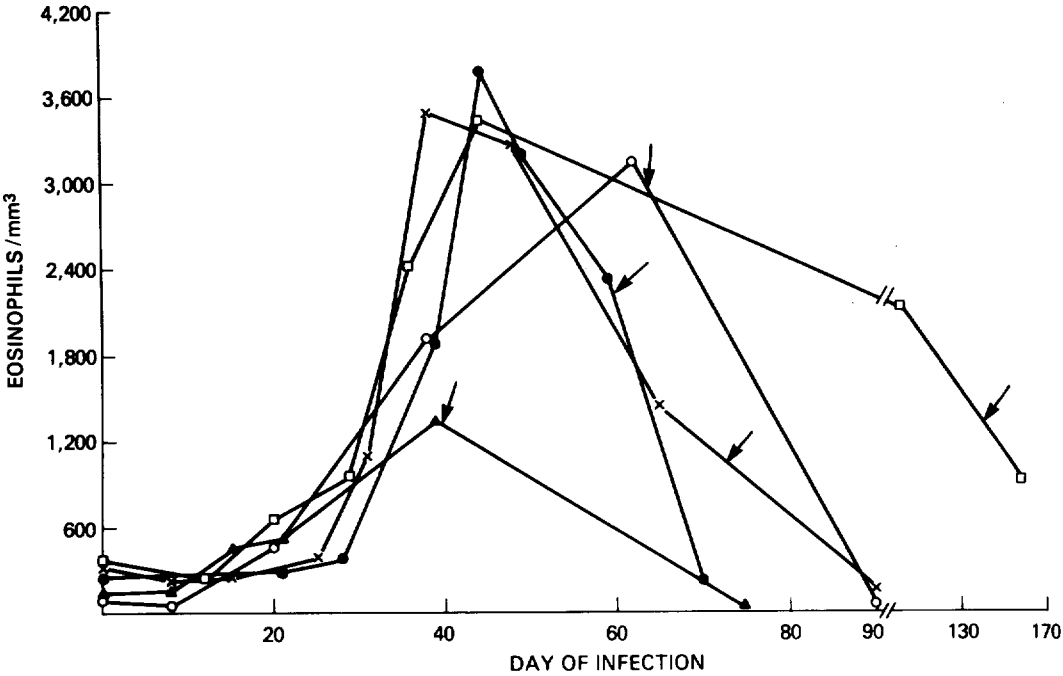

FIGURE 1. Blood eosinophil responses of five normal human volunteers following exposure to *N. americanus* third stage larvae. Arrows indicate treatment with mebendazole. Each symbol represents a different patient.

teers had normal mitogen responses to PHA and Con A initially, and in three there appeared to be some increase in these responses during infection (Fig. 3A). Only two of the four developed any appreciable increase in their responses to hookworm antigen following infection, and in both there were similar increases in responsiveness to tetanus toxoid as well (Fig. 3B). In the most responsive volunteer (Fig. 3B, open squares) maximal responses to all mitogens and antigens studied occurred between days 36–52. However, the reasons for this temporary general enhancement of lymphocyte responsiveness are unclear; extensive studies of sera taken from this and other volunteers at various times post-infection and cultured with both autologous and homologous cells stimulated by mitogens and antigens failed to demonstrate any humoral enhancing or suppressive factors affecting lymphocyte blastogenesis (data not shown).

IgG-specific antibody responses were measured by ELISA in all volunteers. The preinfection values ranged from 0.2–120 U/ml, all but one being within the normal range (66.5 U/ml) determined in 14 North American controls. However, following infection all showed a modest 1.5- to 3-fold rise in IgG antibody titers, and these were seen to peak generally after the third week of infection (Fig. 4).

Total serum IgE was measured by radioimmunoassay in all volunteers. Preinfection values ranged from 100–1,000 ng/ml. In four of the five there was a modest increase of IgE by 5–8 weeks post-infection (Fig. 5). Antibody-specific IgE, also measured by RIA, was found in extremely low quantities. Preinfection values ranged from 4.5–30 ng/ml (normal range 13–40 ng/ml defined in 14 North American controls) (Fig. 5). In only two of the volunteers was there any indication of a rise in antibody level (between days 28 and 38 of infection).

DISCUSSION

The present findings as well as those of others[5, 17] indicate clearly that exposure to a small inoculum of infective third stage hookworm larvae is capable of producing patent infection. Clinically, the effects of this low intensity infection were surprisingly pronounced; immunologically, they were surprisingly minimal. The dermatologic reactions (pruritis and erythema) have

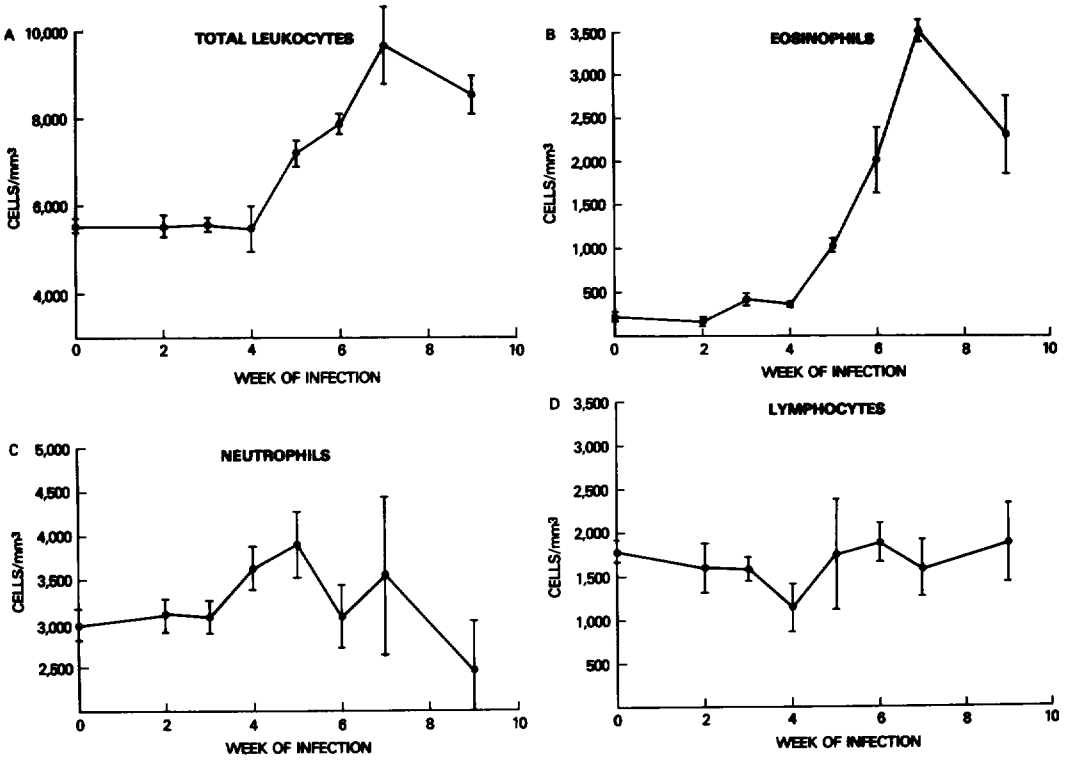

FIGURE 2. Increase in total leukocyte count seen to be maximal about 7 weeks after infection (panel A) is caused entirely by rising eosinophil counts (panel B). Lymphocyte and neutrophil numbers remain essentially unchanged (panels C, D).

been well documented[1, 2, 4, 8] and these were seen in all of our volunteers as well. Pulmonary findings, on the other hand, were negligible; there were no definite symptoms and few positive findings on bronchoalveolar lavage.

Despite the small inoculum, gastrointestinal symptoms were prominent in all volunteers, and in one of these, symptoms were of such severity that treatment was necessary prior to the conclusion of the study. This finding was somewhat surprising since numerous authors[1-5, 8] ascribe gastrointestinal morbidity to a heavy worm burden, noting that many infected people with light worm burdens (primarily from endemic areas) have no symptoms at all. Presumably the naive background of these volunteers afforded them little modulatory protection from the local inflammatory responses initiated by the adult worms in the intestine. The exact mechanisms involved in this local inflammation, however, remain undefined.

Immunologically, the most consistent and dramatic response in all five subjects was the rise in blood eosinophil levels. This rise started consistently after 4 weeks of infection (peaking between days 38 and 64), a period corresponding to the beginning of egg production by mature worms and, interestingly, to the time during which gastrointestinal symptoms developed. Earlier studies in both humans[5-7, 18, 19, 28] and animals have reported similar findings, but not development of such profound eosinophil responses in the face of relatively small inocula of infective larvae or adult worms. Of the various immunologic parameters assessed, the eosinophil response with its presumed T cell dependence[20] was the most conspicuous.

Direct examination of T lymphocyte-mediated responses (assessed by lymphocyte blastogenic techniques) did not yield as clearly defined results. Although all individuals had good responses to the mitogens PHA and Con A as well as to the antigen tetanus toxoid, in only one volunteer was there a notable enhancement of re-

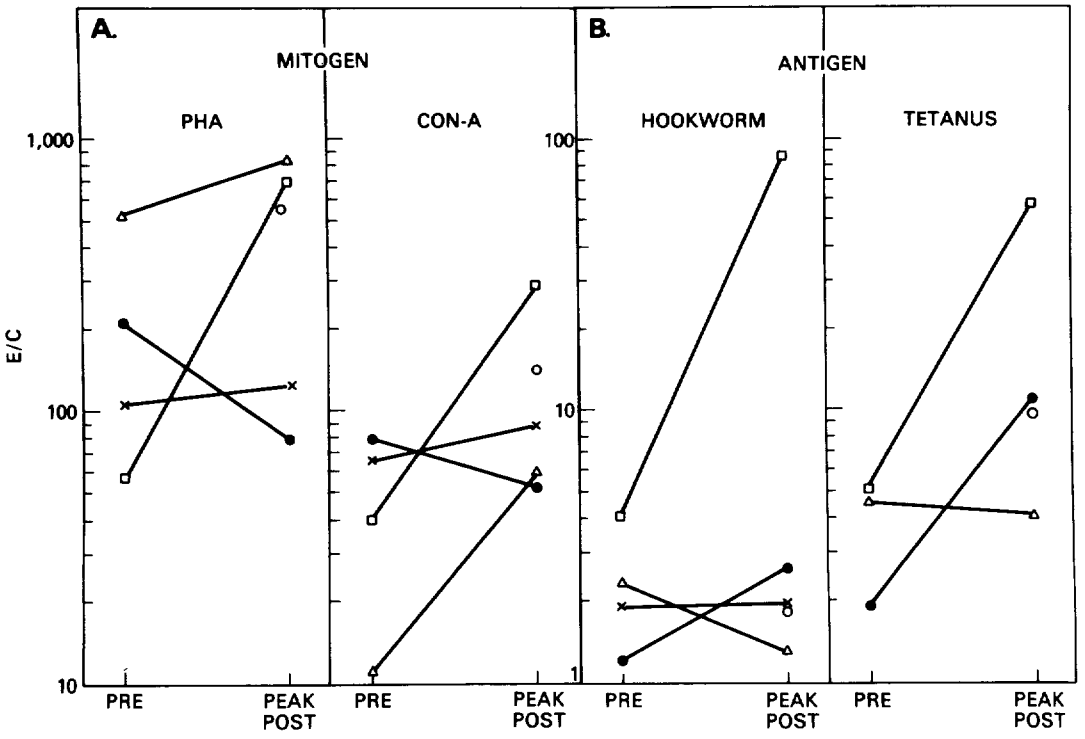

FIGURE 3. Lymphocyte blastogenic response to mitogens (PHA, Con A) and antigens (hookworm, tetanus toxoid) prior to infection (Pre) and at time of peak maximal response after infection (Peak Post). One volunteer (o) did not have preinfection studies. Symbols for each individual are the same as those in Fig 1.

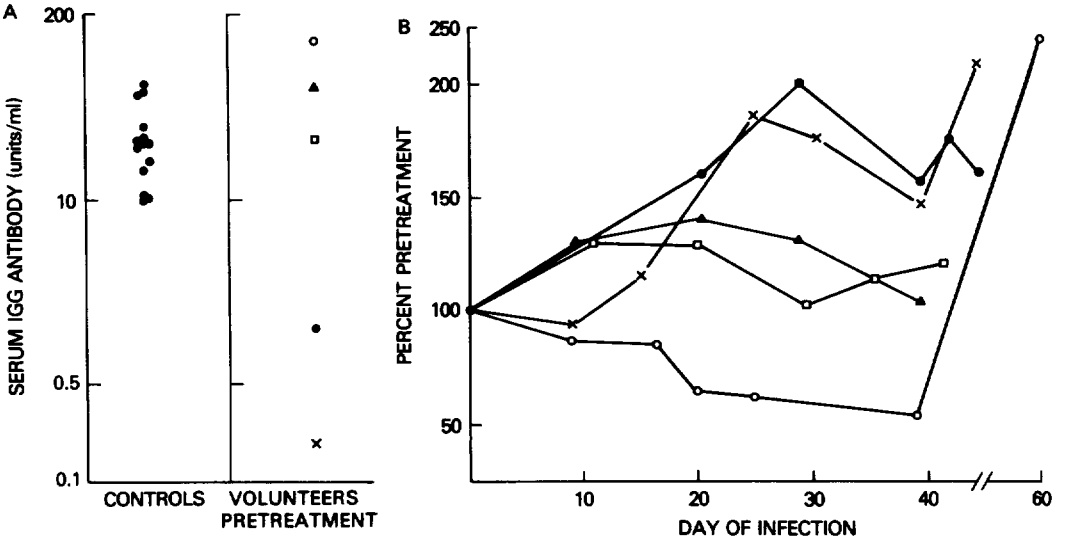

FIGURE 4. **A.** Serum IgG anti-hookworm antibody levels expressed in U/ml measured in 14 normal controls and the five volunteers prior to infection. Symbols for each individual are the same as those in Figs. 1 and 3. **B.** Anti-hookworm IgG antibody response in volunteers expressed as percent of pretreatment values. All 5 volunteers showed an increase in IgG antibodies after week 3 of infection.

sponsiveness to hookworm antigen. The generally poor hookworm antigen responsiveness was surprising since a previous report[9] had indicated significant lymphocyte transformation responses to comparable larval antigen preparations at 17 and 51 days after infection in a subject experimentally infected with 250 larvae of *N. americanus*. Perhaps the more than 5-fold greater infecting dose used in those studies[9] accounts for our discrepant findings. Extensive crossing experiments, using autologous serum, homologous normal serum, and homologous infected serum failed to show the presence of serum suppressive factors that could account for the poor lymphocyte responsiveness to antigen seen in all but one of our volunteers (data not shown). The fact that the hookworm antigen was prepared from larvae and not adults could be important;[19] however, one individual did develop specific antigen responsiveness in this study as did the volunteer in the report from Taylor and Turton.[9] We feel, therefore, that the more likely explanation is a "quantitative" one, reflecting the threshold of detectable blastogenic responsiveness following very light hookworm infection.

Similarly, the total and parasite-specific IgE responses to this low level infection were minimal, though consistent with the findings of others in experimentally induced infections. One report noted that total IgE levels actually fell after the primary infection, increasing only gradually after subsequent infections, and that IgE antibodies were not detected until after the third or fourth infection.[7] These low levels of total IgE seen in our subjects contrast sharply with those seen in individuals from endemic areas[21–27] where much higher IgE levels are found. Such differences, of course, can be accounted for by the heavy repeated infections of individuals in endemic regions and by the inability to exclude concurrent or prior infections of those subjects with other diverse helminthic parasites.

As a model for studying the immunologic aspects of invasive helminth infections in humans, this experimental hookworm infection was rather disappointing, *except* for the very reproducible eosinophilic response. The difficult-to-detect meager IgG, IgE, and lymphocyte blastogenic responses, and the absence of easily demonstrable immunologic suppressor mechanisms make this model unsuitable for analysis of many of the most important immunologic features of the human host response to helminth infections.[29, 30]

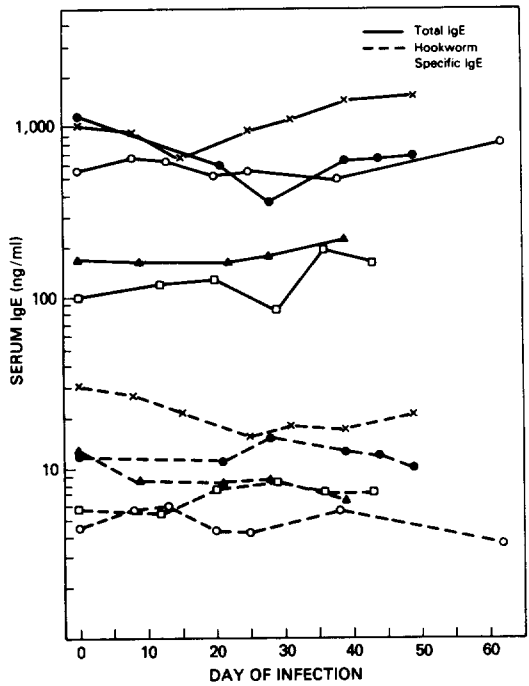

FIGURE 5. Total (——) and parasite-specific (– – –) serum IgE expressed in ng/ml in the five volunteers followed over 8 weeks.

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