
IgG4 responses to antigens of adult *Necator americanus*: potential for use in large-scale epidemiological studies

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Described is an epidemiological investigation of hookworm infections in a rural community in Zimbabwe, where Necator americanus is the only human helminth species present. Among a cohort of 120 individuals the overall prevalence of infection was 78%. Intensity of infection was quantified both as egg counts (range: 0–2563 eggs per g of stool) and worm burden (range: 0–100 worms). Although both these measures provide useful quantitative data, they are tedious to determine in large-scale epidemiological studies and may present social and logistic difficulties. As an alternative screening method, we therefore investigated isotype-specific responses to adult worm antigens of N. americanus. The results show that specific IgG4 responses correlate positively and significantly with both measures of intensity and may be a useful marker of hookworm infection.

Introduction

Hookworm infections caused by *Necator americanus* and *Ancylostoma duodenale* are still widely prevalent, despite the activities of many control programmes. Parasitological screening for hookworm infections is the method of choice for epidemiological studies, and in most investigations the density of eggs in stools has been used as an indirect measure of intensity. More recently, estimates of worm numbers following chemotherapy have been reported as a more direct measure of infection intensity; however, this method suffers from logistic and social difficulties, and its use to date has been limited to a few studies (1–6). The use of other methods, such as serological screening as markers of infection, has not been widely explored despite evidence from a number of studies for a vigorous humoral response to infections in adult volunteers (7–9) and in infected patients (10, 11).

Seroepidemiological studies of the age relationships of serum antibody levels and infection status showed that levels of *N. americanus*-specific antibod-

ies correspond to age-related changes in infection intensity (5, 12, 13).^a

This report describes a study which assesses the relationship between *N. americanus*-specific isotype responses (measured by enzyme-linked immunosorbent assay (ELISA)) and age, sex, and infection status (quantified as the mean number of eggs per g of stool or worm burden) in a village in Zimbabwe with moderate levels of hookworm transmission intensity. The aim of the study was to identify antibody responses to adult worm antigens that may be useful markers of infection in epidemiological screening programmes. Additionally, a method for enhancing the specificity of the assays used in the study was examined.

Materials and methods

Sample collection

The study was conducted in the Charara estate, Kariba, Zimbabwe. The geography and sociodemographic profile of the study population have been described previously (6). Two sequential stool specimens were obtained from a cohort of 120 individuals selected at random in Charara (approximate population, 550) and examined using the Kato technique to estimate the density of eggs. After the

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participants had been treated with albendazole (400 mg single dose; Zentel, SmithKline Beecham, Welwyn Garden City, England), 72-hour stool samples were collected from them. The number of worms expelled was counted to provide an estimate of the worm burden. Pretreatment peripheral blood samples were also collected; the sera were separated from blood samples, aliquoted, and stored in glycerol at -20°C for use in immunoassays.

Antigen preparation

Adult *N. americanus* recovered from the study participants were washed thoroughly in tap water in the field and then frozen at -20°C before being transported on dry-ice to London.

Adult worms were ground to a powder on dry-ice in a mortar and pestle. Antigens that were soluble in the synthetic detergent, *n*-octyl glucoside (NOG) were extracted for 60 min on ice by agitation in 1.5% NOG in phosphate-buffered saline (PBS) containing the protease inhibitors ethylenediaminetetraacetic acid (EDTA) (1 mmol/l), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK; 0.1 mmol/l), phenylmethylsulfonyl fluoride (PMSF; 1 mmol/l) and *N*- α -*p*-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK; 0.2 mmol/l). These inhibitors were used at 1:200 dilution with the parasite extract. The suspension was centrifuged at 26000 g for 30 min at 4°C and the supernatant collected and dialysed against PBS for 48 h with frequent buffer changes to remove the detergent. The protein concentration was estimated by the method described by Bradford (14). The total protein yield was 1–2 mg/ml for each 100 mg of fresh adult worms. The antigen preparation was stored in aliquots of 50–100 μl at -70°C .

Enzyme-linked immunosorbent assay (ELISA)

The optimal concentrations of antigen, antibody, and enzyme-conjugated antibody were determined using checkerboard titrations. The optimal antigen concentration was taken to be the minimum necessary to achieve a condition of "antigen excess". All assays were carried out at a single serum dilution, with the optimal dilution selected for testing being that for which there was good discrimination between positive and negative samples, and at which the positive samples were beginning to titrate out. In all the assays described below, the positive control sera were either pooled from six individuals infected with *N. americanus* with worm burdens >20 or individual sera preselected by screening. The inclusion of positive controls in each run allowed readings to be corrected to a set, reference, positive absorbance. The optimal working dilutions of the

antibody–enzyme conjugate were mainly those recommended by the manufacturers, and if these were inadequate repeats were carried out at other dilutions.

Isotype-specific responses were quantified as absorbances of duplicate samples relative to the positive and negative controls.

To determine the IgA and IgM responses, low-binding Linbro/Titertek plates (Flow Laboratories, Irvine, Scotland) were coated overnight with a 2.5 μg per ml solution of antigen in 0.05 mol/l carbonate buffer (pH 9.6). The plates were blocked for 1 h with 2% (IgA) or 5% (IgM) bovine serum albumin (BSA) in PBS and incubated for 1 h with test sample diluted 1:200 (IgA) or 1:400 (IgM) in PBS/0.05% Tween 20. Peroxidase-conjugated goat antihuman IgA and IgM (Jackson Immuno-research Laboratories, PA, USA) was added to the plates for 2 h at dilutions of 1:10000. The plates were washed three times (IgA) or six times (IgM) with 0.9% saline/0.05% Tween 20 between each incubation step. The reactions were developed using *o*-phenylenediamine/hydrogen peroxide as substrate. After 10 min for the IgM and 30 min for the IgA assays, the reactions were stopped by adding 20 μl of 5 mol/l hydrogen peroxide, and the absorbance was measured at $\lambda = 492\text{ nm}$.

IgG subclass responses were measured by a similar procedure to that described above for IgA, except that for IgG2 the ELISA plates were first coated overnight with poly-L-lysine solution (5 $\mu\text{g}/\text{ml}$) before adding the antigen (10 $\mu\text{g}/\text{ml}$) to facilitate the binding of the relevant carbohydrate antigens. Sera were screened at a dilution of 1:100 for all IgG subclasses, and mouse monoclonal anti-IgG antibodies (Dako Ltd, High Wycombe, England) were added for 3 h at the following dilutions: Pan IgG (1:2000); IgG1 and IgG4 (1:1000); and IgG2 and IgG3 (1:500). This was followed by a further period of overnight incubation at 4°C with peroxidase-conjugated rabbit antimouse IgG (Dako Ltd, High Wycombe, England) at a dilution of 1:1000 for all subclasses.

To measure specific IgE responses, adult antigens (10 $\mu\text{g}/\text{ml}$ solution) were coated onto high-binding Nunc maxisorb plates (Nunc, Kamstrup, Denmark). Rabbit antihuman IgE (Dako Ltd, High Wycombe, England) was used as the first antibody at a dilution of 1:1000, and incubations were carried out for 3 h at room temperature. This was followed by an overnight incubation at 4°C with 1:1000 dilution of a peroxidase-conjugated porcine anti-rabbit antibody (Dako Ltd, High Wycombe, England). The remainder of the assay procedure was carried out in a similar manner to that described above.

Specificity studies

The specificity of the ELISA was assessed by including in each assay sera from eight European adults who had no history of exposure to infection with *N. americanus*. To give an indication of the extent of cross-reaction with other nematode infections and to examine further the specificity of individual IgG assays, each hookworm serum was incubated for 1 h with 25 µg/ml of an *A. lumbricoides* adult worm antigen preparation ("absorbed" sera), and the results compared to sera were treated in a similar manner without prior incubation ("unabsorbed" sera). *A. lumbricoides* was used because its co-existence with *N. americanus* in endemic populations is well documented (15). The adult worm antigens were prepared in a similar manner to that described for *N. americanus*. Serum at a dilution of 1:100 was added to ELISA plates coated with either 2.5 µg/ml of *A. lumbricoides* or *N. americanus* adult worm antigen preparations. A peroxidase-labelled rabbit antihuman IgG conjugate (Dako Ltd, High Wycombe, England) was used at a dilution of 1:1000. The rest of the assay procedure was similar to that described above.

Statistical analyses

All statistical analyses were carried out using the Complete Statistical Software Package (StatSoft, Inc., OK, USA). Because the distribution of the isotype responses was skewed, nonparametric Mann-Whitney *U* tests were used to compare values between sexes.

Spearman's rank correlation was used to assess the strength of association between age, isotype levels, and levels of infection, with the results being expressed as a coefficient of rank correlation.

Results

Parasitology

All the worms recovered were identified as *N. americanus*. The overall prevalence of *N. americanus* infection in Charara was 78%; the egg count range was 0–2563 eggs per g (epg) and the worm load, 0–100 worms. The two methods used to estimate intensity were strongly associated (Spearman's $r^2 = 0.47$, $P < 0.001$; $n = 121$).

The results of Spearman's rank correlation analyses revealed a significant positive association

between age and epg (Spearman's $r^2 = 0.30$, $P < 0.001$; $n = 119$) or worm load (Spearman's $r^2 = 0.22$, $P < 0.05$; $n = 117$). Males ($n = 58$) carried significantly heavier worm loads and egg counts (2.8 and 212.4, resp.) than females ($n = 62$) (1.9 and 137, resp. Mann-Whitney *U* test for comparison with males, $P < 0.01$ and $P < 0.05$, resp.).

Correlation between host infection status and isotype responses

The correlations between isotype responses and epg and worm loads are shown in Table 1. Positive correlations were found between egg counts and Pan IgG, IgG2, IgG3, IgG4, and IgE for the total study population. Correlations with worm loads were similar, with the exception of IgG3, for which there was no correlation. The relationships between IgG4 and intensity levels are shown in Fig. 1 (egg counts) and Fig. 2 (worm load).

Table 1: Spearman's rank correlation coefficients between isotype responses (absorbances) and density of eggs per g of stool (epg) or worm load for the study population ($n = 120$), Charara, Zimbabwe

Isotype	Hookworm infection level:		
	egg	Worm load	Age
Pan IgG	0.30 ^a	0.28 ^b	0.30 ^a
IgG1	0.10 ^c	0.09 ^c	-0.01 ^c
IgG2	0.20 ^d	0.23 ^d	0.14 ^c
IgG3	0.20 ^d	0.18 ^c	0.30 ^a
IgG4	0.39 ^b	0.28 ^b	0.31 ^a
IgE	0.25 ^b	0.28 ^b	0.17 ^c
IgA	0.03 ^c	-0.11 ^c	0.25 ^b
IgM	0.01 ^c	-0.15 ^c	-0.12 ^c

^{a-d} Spearman's rank correlation coefficient: $P < 0.001$, $P < 0.01$, $P > 0.05$ (not significant), $p < 0.05$, resp.

Fig. 1. Plot showing the relationship between egg counts and optical absorbance levels of IgG4 responses to adult *Necator americanus* antigens.

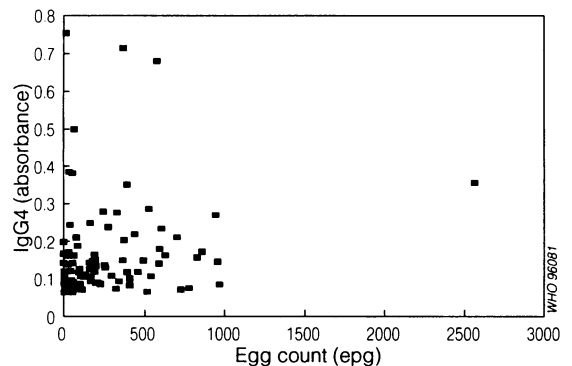


Fig. 2. Plot showing the relationship between worm burden and optical absorbance levels of IgG4 responses to adult *Necator americanus* antigens.

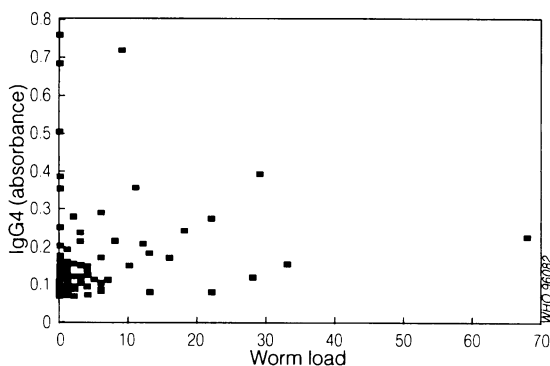


Table 2 shows that IgG4 levels were significantly greater among males who had higher infection levels; IgM levels were significantly lower among this group.

Correlation studies between host age and isotype responses

Correlations of isotype responses with age are shown in Table 1. Significant positive correlations with age were found for Pan IgG, IgG3, IgG4, and IgA.

Specificity studies

The mean IgG optical absorbances for unabsorbed and absorbed responses to *A. lumbricoides* and *N.*

Table 2: Comparison of Mann-Whitney *U* tests for isotype responses, by sex, among the study population

Isotype	Mean absorbance for:		Z statistic ^a
	Females (n = 58)	Males (n = 62)	
Pan IgG	1.05 (0.50) ^b	1.19 (0.58)	-1.7 ^c
IgG1	0.31 (0.24)	0.35 (0.30)	-0.6 ^c
IgG2	0.10 (0.06)	0.13 (0.12)	-0.6 ^c
IgG3	0.19 (0.19)	0.14 (0.05)	-0.2 ^c
IgG4	0.13 (0.10)	0.17 (0.16)	-2.1 ^d
IgE	0.17 (0.05)	0.22 (0.15)	-1.7 ^c
IgA	0.52 (0.24)	0.58 (0.36)	-0.1 ^c
IgM	0.69 (0.23)	0.54 (0.25)	-4.1 ^e

^a Normal approximation to the Mann-Whitney test statistic adjusted for ties.

^b Figures in parentheses are standard deviations.

^c Not significant ($P > 0.05$).

^d $P < 0.05$.

^e $P < 0.001$.

Table 3: Mean IgG responses to *Necator americanus* and *Ascaris lumbricoides* antigens before and after absorption with adult *A. lumbricoides* NOG antigens, among 123 individuals, Charara, Zimbabwe

ELISA carried out with:	Mean IgG absorbance
<i>Necator</i> antigens and unabsorbed sera	0.76 (0.77) ^a
<i>Necator</i> antigens and absorbed sera	0.58 (0.52)
<i>Ascaris</i> antigens and unabsorbed sera	0.60 (0.47)
<i>Ascaris</i> antigens and absorbed sera	0.16 (0.13)

^a Figures in parentheses are standard deviations.

americanus are shown in Table 3. Both anti-IgG *Ascaris* and anti-IgG *Necator* responses were significantly reduced by absorption (Wilcoxon's matched pair test: $Z = 4.6$, $P < 0.001$; $n = 120$; and $Z = 8.9$, $P < 0.001$; $n = 120$, resp.). However, absorption of sera with *A. lumbricoides* significantly depleted the specific IgG response to *Ascaris* antigens, suggesting that the responses to *Necator* antigens may not be antigen related.

Discussion

Investigated was the use of specific antibody responses to adult *N. americanus* antigens as a screening tool for epidemiological studies. Parasitological data (egg counts and worm loads) were obtained from field studies conducted in the rural community of Charara, in Kariba, Zimbabwe, where *N. americanus* was the only human helminth species present. Except for IgG2, there were modest increases in the levels of all isotypes measured in infected individuals. Pan IgG, IgG2, IgG4, and IgE responses were positively correlated with both egg counts and numbers of worms expelled; and intensity levels increased with age and were significantly higher among males. Correlation studies between isotype levels and these variables indicated that only IgG4 responses were both significantly associated with age and sex. The levels of this isotype were significantly higher among more intensely infected males. The strength of these associations suggests that determination of IgG4 levels could be useful in large-scale screening programmes.

A functional role for isotype responses in *N. americanus* infections has yet to be described. As far as IgG4 is concerned, there is growing evidence for a restricted IgG4 subclass response following chronic stimulation in a number of helminth infections (16, 17). In contrast to the other IgG subclasses, IgG4 responses do not participate in complement fixation.

However, unlike the other IgG subclass antibodies, IgG4 antibodies can sensitize mast cells and basophils and participate in immediate hypersensitivity reactions. This may be of some relevance in helminth infections (18).

The specificity of the assays was tested by including negative, uninfected sera in all runs. The absorbance readings obtained for these sera in most assays (except for IgG2) were well below the mean levels obtained for the Zimbabwean study samples (Table 4). The specificity was further assessed by screening the Zimbabwean study sera on ELISA plates coated with adult *A. lumbricoides* antigens. Preliminary results obtained for specific IgG responses showed dual recognition of both *N. americanus* and *A. lumbricoides* antigens, suggesting the existence of cross-reactive antigens. Depletion experiments were carried out by preabsorbing infected sera with adult *A. lumbricoides* antigen prior to screening with adult *N. americanus* antigens; this significantly reduced IgG responses to *A. lumbricoides* but not to *N. americanus* antigens. These experiments need to be repeated in an IgG4 assay but we were unable to carry this out because of lack of antigen material. The specificity of hookworm assays would probably be enhanced by initially preabsorbing infected samples with a cocktail of antigens from common helminth infections prior to performing the ELISA.

In conclusion, we have described a rapid and more socially acceptable method of assessing populations at risk from hookworm infections using immunological markers of infection. Levels of IgG4 responses to antigens of adult *N. americanus* correlated positively with infection levels and age among an infected Zimbabwean community. IgG4 levels were also significantly higher among males who were more heavily infected.

Table 4: Mean isotype responses of negative controls and infected individuals in Charara, Zimbabwe

Isotype	Mean absorbance:	
	Study population (n = 120)	Negative controls (n = 8)
Pan IgG	1.14 (0.53) ^a	0.34 (0.09)
IgG1	0.30 (0.23)	0.13 (0.06)
IgG2	0.11 (0.07)	0.12 (0.02)
IgG3	0.17 (0.14)	0.13 (0.02)
IgG4	0.15 (0.12)	0.09 (0.003)
IgE	0.19 (0.09)	0.16 (0.19)
IgA	0.54 (0.28)	0.18 (0.05)
IgM	0.59 (0.26)	0.21 (0.04)

^a Figures in parentheses are standard deviations.

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Résumé

Réponses en IgG4 aux antigènes des formes adultes de *Necator americanus*: possibilités d'applications dans les études épidémiologiques à grande échelle

Les infestations dues à *Necator americanus* et *Ancylostoma duodenale* sont toujours prévalentes dans les pays en développement malgré les activités des programmes de lutte. Jusqu'à maintenant, les études épidémiologiques portant sur ces infestations ont été fondées sur des méthodes parasitologiques et sur le dépistage à grande échelle dans les populations. Ces investigations, bien qu'ayant fourni des données quantitatives sur la prévalence et l'intensité de ces parasitoses, sont souvent fastidieuses et peuvent poser des problèmes sociaux et logistiques. Pour tenter d'éviter ces inconvénients, nous avons recherché si l'utilisation des réponses en anticorps aux antigènes des formes adultes de *N. americanus* pouvait être utilisée comme méthode de dépistage.

L'étude a été réalisée dans une communauté rurale vivant de l'agriculture à Kariba, au Zimbabwe. *N. americanus* est la seule espèce d'helminthe ayant une prévalence élevée (78%) dans la région, où les taux d'infestation sont modérés. Les vers adultes utilisés comme source d'antigènes ont été obtenus chez des sujets infestés après administration d'un vermifuge. Les résultats montrent que l'infestation entraîne une production accrue de tous les isotypes, à l'exception des IgG2. Les réponses multiples en IgG, IgG2, IgG4 et IgE présentaient une corrélation positive et significative avec les numérations d'œufs et la charge en vers. L'infestation étant fonction de l'âge et du sexe des sujets participant à l'étude, les taux d'isotypes ont également été examinés en fonction de ces variables. Les taux globaux d'IgG, IgG3, IgG4 et IgA étaient associés de façon positive et significative avec l'âge; cependant, seules les réponses en IgG4 et en IgM différaient significativement selon le sexe. Les taux d'IgG4 étaient sensiblement plus élevés chez les sujets de sexe masculin les plus fortement parasités, alors que, dans ce groupe, les taux d'IgM

étaient significativement plus faibles. D'après ces résultats, les réponses en IgG4 aux antigènes des formes adultes de *N. americanus* pourraient utilement servir de marqueurs de l'infestation dans les études en population. L'article décrit également une méthode permettant d'augmenter la spécificité de l'épreuve. Avant d'effectuer une épreuve, le sérum du sujet infesté est préabsorbé en présence d'antigènes adultes d'*A. lumbricoides*, vers absents de la population étudiée, mais dont certains antigènes donnent des réactions croisées avec ceux de *N. americanus*. Les résultats semblent indiquer que cette approche réduit les réponses aux antigènes communs, mais non aux épitopes spécifiques de *Necator*.

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