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Repeated albendazole treatments enhance Th2 responses to *Ascaris lumbricoides* but not aeroallergens in children from rural communities in the Tropics

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Abstract

Geohelminth infections are associated with a modulation of immunity to parasite antigens and aeroallergens that may be affected by anthelmintic treatment. To investigate this, we compared cytokine responses between children that had received repeated doses of albendazole over a year or no treatment. Whole blood was cultured with *Ascaris* antigen and house dust mite and cockroach allergens and IL-5, IL-13, IFN-γ, and IL-10 were measured. Anthelmintic treatment was associated with enhanced production of Th2 cytokines to parasite antigen, but did not affect responses to aeroallergens. The data indicate that long-term treatment may be associated with increased antiparasite Th2 immunity.

Keywords

geohelminths; *Ascaris lumbricoides*; allergy; atopy; aeroallergens; Th2 cytokines; immune modulation

Introduction

Geohelminth parasites are estimated to infect 2 billion humans worldwide, 1 and infections induce strong immunological responses associated with the type 2 cytokines IL-4, IL-5, and IL-13.2,3 Geohelminth infections may have major modulatory effects on host immunity, 4,5 and explain partly the low prevalence of allergic diseases reported in the rural Tropics. 5

Previous studies have shown that single or multiple doses of anthelmintic drugs have negligible effects on human cytokine responses to parasite antigens,6,7 and that long-term anthelmintic treatments may increase allergen skin test reactivity to aeroallergens.8,9 Because control geohelminth programs administer anthelmintic drugs periodically over long periods, it will be important to determine if anthelmintics administered over the long-term have important immunologic effects.

To investigate if repeated anthelmintic treatments have important effects on immunity to parasite antigens and aeroallergens, we examined cytokine responses and histamine release

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in whole blood from children living in rural Ecuadorian communities that had received albendazole every 2 months for a year or no treatment.

Materials and Methods

Study design and subjects

Children attending schools in Pichincha Province in Ecuador were sampled within a cluster-randomized study that examined the effect of albendazole on the prevalence of allergy. The study design is described elsewhere.10 Briefly, children attending 68 rural schools were recruited. Schools were randomized to receive either albendazole (single doses of 400 mg every 2 months for 12 months [total of 7 treatments] or no treatment). Albendazole treatments were directly observed. The present study was a cross-sectional study nested within the intervention study and was performed at the end of 12 months follow-up. A total of 214 children from 42 schools were selected from 1,632 children that completed follow-up. Children were selected blind to school treatment status but such that allergen skin test positive children were over-represented in the sample (2:3 ratio of skin test positive to skin test negative children). No tissue helminth infections or malaria were endemic in these communities. Informed written consent was obtained from a parent of each child. The study protocol was approved by the ethics committees of the Hospital Pedro Vicente Maldonado, Ecuador, and St George's Hospital, UK.

Allergen skin prick testing, and sample collection and analysis

Skin prick testing to house dust mite (*Dermatophagoides pteronyssinus*; Greer Laboratories), American cockroach (*Periplaneta americana*, Greer Laboratories), *Alternaria tenuis* (Greer Laboratories), cat (Greer Laboratories), grass pollen mix (Greer Laboratories), fungi pollen (Greer Laboratories), histamine (ALK-Abello), and saline (ALK-Abello) controls was performed as described previously.10 Reactions were considered positive if the mean wheal diameter was at least 3 mm greater than saline. Stool samples were collected from children at the beginning of the study (i.e. before receiving the 1st dose of albendazole) and at 12 months (i.e. before receiving the 7th dose of albendazole) and were examined using the modified Kato-Katz and formol-ethyl acetate concentration methods.11 Blood samples (7 mL) were drawn into Vacutainers (Becton Dickinson) containing sodium heparin 7 days after receiving the 7th dose of albendazole. Blood samples were transported in insulated boxes at ambient temperature and analysed within 5 hours of collection.

Whole blood cultures

Whole blood was diluted 1 in 4 in RPMI 1640 (BioWhittaker) containing L-glutamine, 80 mg/ml gentamicin and 1% HEPES. Diluted whole blood (0.5 mL) was cultured alone or in the presence of *A.lumbricoides* adult worm antigen,2 lipopolysaccharide (LPS; Sigma-Aldrich), tuberculin (PPD; Statens Serum Institute), *Staphylococcus* enterotoxin B (SEB; Sigma-Aldrich), all at 10 μg/mL, and *D.pteronyssinus* (Greer Laboratories) (100 AU/mL), and *P.americana* allergen extract (Greer Laboratories) (1/50 dilution). Cultures were incubated in a humidified atmosphere of 5% CO_2 at 37°C.

Cytokine, antibody, and histamine assays

Supernatant fluids were collected from cultures at 24 hours (IL-10) and 5 days (IFN-□, IL-5, and IL-13) and stored in liquid nitrogen. Cytokine levels were measured using antibody pairs (BD Biosciences) by sandwich ELISA following the manufacturers instructions. The lower detection limits for IL-5, IL-13, IL-10 and IFN-γ were 7.8, 58.6, 19.5, and 19.5 pg/mL, respectively. Total IgE levels were measured as described previously. 11 Histamine release assays to *Ascaris* adult and larval stage (L2/L3 and L3/L4) antigens,2

and aeroallergens (*D.pteronyssinus* and *P.americana* [Greer Laboratories], purified Der p1 and recombinant Der p2 [Indoor Biotechnologies]), all at concentrations of 0.03 μg/mL, were performed as described11 using a commercial assay (Immunotech)

Statistical analysis

Cytokine levels and percent histamine release by treatment or infection group were compared using the ranksum test. Proportions were compared using the chi-squared test. Associations between cytokines were assessed by calculation of Spearman's rank correlation coefficients. Significant findings for a treatment effect were assessed using multivariate linear regression (using $\log_e$-transformed cytokine levels) or logistic regression (cytokine responders vs. non-responders) in which *a priori* confounders and relevant baseline factors, and clustering by school, were controlled for in the analysis. The interaction between atopy and treatment group on cytokine levels was assessed by addition of an interaction term to linear regression models for the cytokines. Statistical significance was inferred by $P \leq 0.01$ for bivariate analyses to minimize Type 1 statistical errors. Analyses were done with Stata 7 (Stata Corporation).

Results

Study population

A total of 214 children were investigated of which 107 received no study anthelmintic treatment and 107 received study treatment. Baseline characteristics of the children are shown in Table 1 for which there were no significant differences between treated and untreated children.

Cytokine production

Treatment was associated with greater production of IL-5 and IL-13 by *A.lumbricoides*-stimulated (IL-5: bivariate, P<0.0001; multivariate; P=0.005. IL-13: bivariate, P=0.01; multivariate, P=0.02) and SEB-stimulated (IL-5: bivariate, P<0.0001; multivariate, P=0.03. IL-13: bivariate, P=0.01; multivariate, P=0.13) cultures (Table 2). SEB-stimulated and A.lumbricoides-stimulated IL-5 (rho=0.52, P<0.0001) and IL-13 (rho=0.40, P<0.0001) were strongly correlated. The treatment effect for parasite-antigen and SEB-induced IL-5 and IL-13 was not modified by allergen skin test status. Treatment was associated with a reduction in the production of IL-10 (bivariate, P=0.004; multivariate, P=0.007) by *A.lumbricoides*-stimulated cultures and also in the proportion of children (univariate, P=0.004; multivariate, P=0.02) producing detectable levels of IL-10. Levels of *A.lumbricoides*-induced IL-10 were not associated with levels of IL-5 or IL-13. There were no differences in levels of IFN-γ or the proportions of individuals producing detectable levels of IFN-γ to any of the stimuli between the treatment groups. Cytokine levels in cultures stimulated with *D.pteronyssinus* and *P.americana* antigens did not differ significantly between treatment groups. Cytokine production in antigen and SEB-stimulated cultures did not differ significantly by geohelminth infection status in treatment and no treatment groups (data not shown).

Histamine release

Histamine release to parasite antigens and aeroallergens did not differ significantly between treatment groups (Table 2). Histamine release (>10%) was strongly associated with skin test reactivity for *D.pteronyssinus* (χ^2=13.5, P<0.001) and *P.americana* (χ^2=11.5, P=0.001).

Discussion

Our data, from a cross-sectional analysis of school children living in rural Ecuadorian communities that are endemic for ascariasis and trichuriasis, suggest that repeated anthelmintic treatments cause significant increases in the Th2 cytokines, IL-5, and IL-13, by peripheral blood leukocytes (PBLs) stimulated with *A.lumbricoides* antigen, and to a superantigen stimulus with *Staphylococcus* enterotoxin-B (SEB). Because levels of *A.lumbricoides* and SEB-induced Th2 cytokines were strongly positively associated, it is likely that the enhanced superantigen response was at least partially attributable to the elevated antiparasite cytokine response. There was no strong evidence for alterations in the host cytokine or IgE-mediated inflammatory responses (measured by histamine release) to aeroallergens (*D.pteronyssinus* and *P.americana*), or other heterologous immunological stimuli (tuberculin and LPS) in treated compared to untreated children. The data provides evidence that long-term anthelmintic treatment has important effects on antiparasite immunity but not on aeroallergen-associated immunity in peripheral blood.

A strength of the study was that selection into the study was blind to treatment status and the two treatment groups were balanced with respect to important baseline factors. A relatively large sample of children was investigated optimizing the ability of the study to detect important differences. Children were selected from a large number of schools thus minimizing any systematic biases that could occur by sampling a few schools. The data was collected in a population where ascariasis and trichuriasis are of high prevalence (defined as ≥50%12) and light intensity (defined as >90% of individuals with stool egg counts of <50,000 eggs per gram (epg) and <10,000 epg for *A.lumbricoides* and *T. trichiura*, respectively12). The immune modulating effects in populations with heavy infection intensities may be greater or the effects may be different for other geohelminth parasites such as hookworm. Few populations in Latin America have high prevalence/heavy intensity infections with ascariasis and trichuriasis because of widespread access to anthelmintic drugs and mass treatment programs, and our findings may be generalizable to most endemic areas where these parasites predominate. Repeated albendazole treatments did not cure all geohelminth infections in the treatment group and the prevalence of geohelminth infections declined slightly in the no treatment group at 12 months - sub-optimal treatments and possible treatment contamination in the treatment and no treatment groups, respectively, may have attenuated immunological differences between the groups. Blood samples were analyzed 7 days after receiving the final albendazole treatment, but it is unlikely that recent treatment would affect cytokine responses because most treated children were infection-free at this time and we have shown previously that antiparasite cytokine responses do not alter up to 35 days after treatment.6

Enhanced Th2 responses to *A.lumbricoides* antigen were not associated with changes in Th2 immune responses to the aeroallergens, *D.pteronyssinus* and *P.americana*, that are the important allergens associated with allergen skin test reactivity in this population.10,11 Previous intervention studies have provided evidence for increased allergen skin test reactivity after repeated anthelmintic treatments.8,9 A mechanism by which this could occur is the reversal of geohelminth-mediated suppression of allergic reactivity accompanied by an increased production of aeroallergen-induced Th2 cytokines and associated allergic inflammation. Such suppression has been suggested to occur through the enhanced production of parasite-antigen induced IL-10.13 In this study, we did not observe an effect of anthelmintic treatment on Th2 cytokine production or IgE-mediated inflammation to aeroallergens. Overall the data provides biological support for the clinical observations from the randomized intervention study in which the current study was nested, of a lack of effect of repeated anthelmintic treatment on the prevalence of skin test reactivity and parameters of clinical allergy.10

Suppression of Th2 cytokine responses in infected individuals may be an important survival mechanism for geohelminths. Several studies have provided evidence that Th2 cytokines mediate protective immunity against geohelminths in humans.3,14,15 The data from this study indicate that continuous exposure to geohelminths (i.e. the non treatment group) may suppress parasite-specific Th2 cytokine production and that repeated treatments with albendazole over a period of a year may reverse this effect. Interestingly, IgE-mediated inflammatory responses (measured by histamine release) to parasite antigens were not affected by long-term treatment. Previous studies examining the short-term effects of two or more doses of anthelmintic drugs on the immune response to ascariasis6 and hookworm7 showed negligible effects on cytokine productions by parasite antigen-stimulated peripheral blood mononuclear cells. There are three possible explanations for the differences in short and long term immunologic effects of anthelmintics. Firstly, a prolonged infection-free period may be required to reverse the suppression of Th2 cytokines. Secondly, geohelminth larvae may be the primary target of host immunity and immune regulation.6,15 In this study, the families of children in the treatment group received albendazole every two months. If suppression of Th2 responses was linked to larval infections with *A.lumbricoides*, then the reversal of suppression could be delayed if the infection reservoir in families was eliminated gradually. Finally, albendazole *per se* may stimulate Th2 cytokine responses in the long-term although there is no evidence to support this.

In conclusion, our data provide evidence that repeated treatments with albendazole enhance Th2 cytokine production by PBLs stimulated with *A.lumbricoides* antigen in school children living in high prevalence communities for ascariasis and trichuriasis. The data do not support a role for geohelminths in mediating important systemic effects on immunity to aeroallergens of other heterologous antigens, or at least those that may be altered by anthelmintic treatment. Our findings do not preclude, however, subtle systemic immunological effects or effects localized to particular tissues such as the lungs or intestine.

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Table 1

Variable	No treatment (N=107)	Treatment (N=107)
Age Mean (SD)	9.7 (1.8)	9.3 (1.9)
Sex Male/Female	54/53	54/53
Socioeconomic level Mean (SD)	2.1 (1.0)	1.9 (0.9)
Crowding (persons/rm) Mean (SD)	2.6 (1.2)	2.4 (1.1)
BMI Mean (SD)	17.3 (2.5)	16.7 (2.1)
Total IgE (IU/mL) GM, t=0 GM, t=12	1,208 1,110	953 687
White cell count ($\times 10^6$ cells/L) Mean (SD)	7,599 (1,578)	7,705 (1,664)
Allergen skin test reactivity, t=12 Any *D.pteronyssinus* *P.americana* [a]Others	37.4% 18.7% 29.9% 0.9%	37.4% 14.0% 30.8% 1.9%
Geohelminth infections, t=0 Any *A.lumbricoides* [b]Intensity, GM (range) epg *T. trichiura* [b]Intensity, GM (range) epg Hookworm *S. stercoralis*	(N=106) 75.5% 56.6% 5,500 (71-294,211) 57.1% 437 (71-23,031) 5.7% 0%	(N=107) 74.8% 57.9% 6,819 (71-213,641) 57.9% 573 (71-64,681) 9.4% 4.7%
Geohelminth infections, t=12 Any *A.lumbricoides* [b]Intensity, GM (range) epg *T. trichiura* [b]Intensity, GM (range) epg Hookworm *S. stercoralis*	(N=99) 60.6% 34.3% 7,621 (211-268,661) 51.5% 463 (71-36,751) 3.0% 0%	(N=102) 21.6% 5.9% 997 (71-14,001) 19.6% 99 (71-3,991) 0% 0%
[c]Treatments 0 1-3 4-6 7	100% 0% 0% 0%	0 1.9% 8.4% 89.7%

Characteristics of the 214 children recruited that either received albendazole treatments (107 children) or no treatment (107 children). GM - geometric mean. SD - standard deviation. Epg - eggs per gramme of stool. t - time of observations (t=0, baseline or pre-treatment; t=12, at 12 months of follow-up)

[a]Includes positive allergen skin tests to *A. tenuis*, cat, grass pollen mix, and fungimix.

[b]Infection intensities calculated excluding non-infected children.

[c]Number of directly observed treatments with single doses of 400 mg of albendazole

Table 2

Immunologic variable	No Treatment			Treatment		
	N	Median (IQR)	R,%	N	Median (IQR)	R,%
Cytokine						
IL-10						
Medium	102	0 (0-0)	20	95	0 (0-0)	13
SEB	102	131 (86-217)	92	95	116 (55-212)	97
Ascaris	102	0 (0-8) *	27 *	95	0 (0-0)	9
PPD	102	0 (0-18) *	34	95	0 (0-0)	20
LPS	102	1,503 (1,1016-2,035)	100	95	1,284 (817-1,949)	100
Cockroach	102	14 (0-50)	64	95	22 (0-122)	61
HDM	102	0 (0-221)	50	95	0 (0-278)	43
IFN-□						
Medium	107	0 (0-0)	15	107	0 (0-0)	11
SEB	107	9,961 (9,160-9,961)	100	107	9,961 (9,257-9,961)	100
Ascaris	107	0 (0-2)	28	107	0 (0-28)	32
PPD	107	72 (4-389)	77	107	107 (3-521)	79
LPS	107	68 (0-407)	74	107	71 (0-402)	69
Cockroach	107	0 (0-8)	29	107	0 (0-0)	20
HDM	107	0 (0-90)	59	107	35 (0-172)	61
IL-5						
Medium	107	0 (0-0)	18 ***	92	0 (0-0)	2
SEB	107	1,374 (850-1,902) ***	100	92	1,992 (1,274-1,992)	100
Ascaris	107	11 (0-69) *	60	92	43 (0-173)	74
PPD	107	4 (0-33)	56	92	3 (0-38)	55
LPS	107	0 (0-0)	15	92	0 (0-0)	5
Cockroach	107	0 (0-0)	16	92	0 (0-0)	12
HDM	107	0 (0-0)	8	92	0 (0-0)	5
IL-13						
Medium	104	0 (0-0)	22	106	0 (0-8)	30
SEB	104	4,274 (2,929-6,991) *	100	106	5,919 (3,651-7,440)	100
Ascaris	104	18 (0-141) *	65	106	84 (0-1279)	75
PPD	104	10 (0-124)	59	106	10 (0-92)	58
LPS	104	0 (0-2)	28	106	0 (0-3)	28
Cockroach	104	0 (0-1)	26	106	0 (0-2)	30
HDM	104	0 (0-0)	23	106	0 (0-4)	35
Histamine release						
Ascaris antigen						
Ascaris adult	32	39 (3-69)	69	19	47 (9-94)	74
Larval L2/L3	32	28 (1-100)	94	19	75 (42-108)	84

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Immunologic variable	No Treatment			Treatment		
	N	Median (IQR)	R,%	N	Median (IQR)	R,%
Larval L3/L4	32	95 (62-100)	91	19	100 (71-100)	89
Aeroallergen						
D.pteronyssinus	32	1 (0-3)	15	19	0 (0-2)	6
Der p1	32	0 (0-2)	7	19	0 (0-6)	11
Der p2	32	0 (0-3)	3	19	0 (0-1)	0
P.americana	32	3 (1-10)	27	19	2 (2-8)	17

Cytokine levels and histamine release among the children that either received albendazole treatments (107 children) or no treatment (107 children) at the end of the 12-month observation period. Sample sizes (N) for cytokine and histamine release assays are shown. IQR-interquartile range. R (%) - proportion of individuals with detectable cytokine levels or with >10% histamine release. P values are from bivariate analyses:

* - P≤0.01

** - P≤0.001

*** - P≤0.0001.