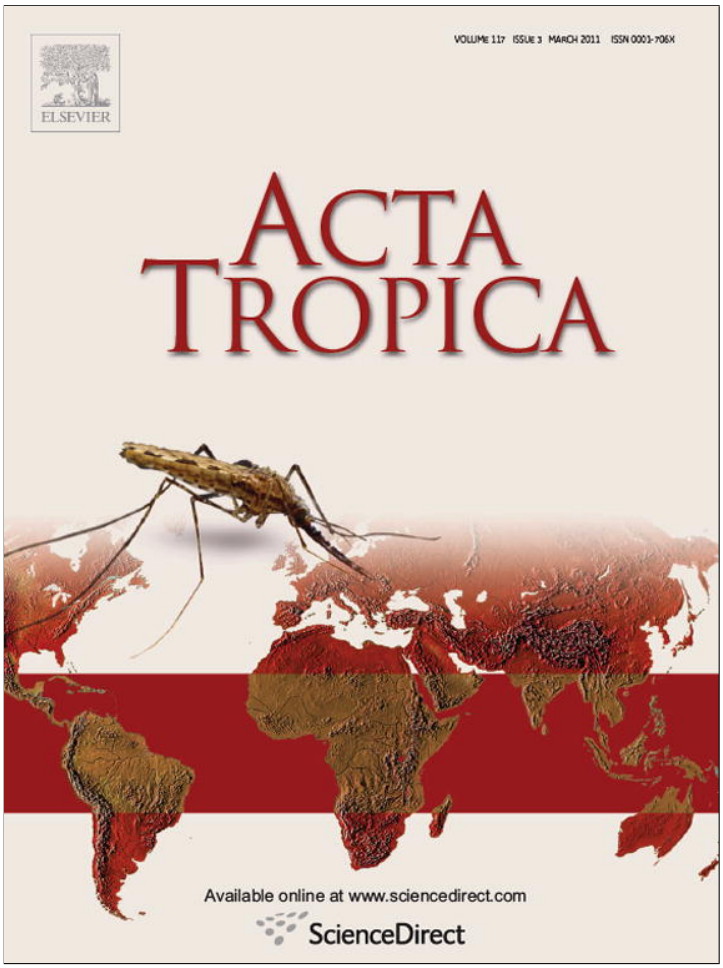

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Co-infection with *Ascaris lumbricoides* modulates protective immune responses against *Giardia duodenalis* in school Venezuelan rural children

I. Hagel[a,*], M. Cabrera[a], F. Puccio[a], C. Santaella[a], E. Buvat[a], B. Infante[b], M. Zabala[b], R. Cordero[c], M.C. Di Prisco[a]

[a] Instituto de Biomedicina, Universidad Central de Venezuela, Ministerio de Salud, Venezuela
[b] Escuela de Nutrición, Universidad Central de Venezuela, Venezuela
[c] Escuela de Bioanalisis Facultad de Medicina, Universidad Central de Venezuela, Venezuela

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ABSTRACT

We evaluated the effect of *Ascaris lumbricoides* on *Giardia duodenalis* infection and TH1/TH2 type immune mechanisms toward this parasite in 251 rural parasitized and 70 urban non-parasitized school children. The children were classified according to light (0–5000 eggs/g faeces) or moderate (>5001–50,000 eggs/g faeces) *A. lumbricoides* infection. Anti *G. duodenalis* skin hyper-reactivity, IgE, IgG, IL-13, IFN γ, IL6 and IL-10 levels were compared among *G. duodenalis* infected and non-infected children according to light or moderate *A. lumbricoides* infection. It was found that 62% of the *A. lumbricoides* moderately infected children were co-infected by *G. duodenalis* compared to 45% of the lightly infected group. After treatment, 42% of the *A. lumbricoides* moderately group were infected with *G. duodenalis* compared to 11% of their lightly counterparts, being *A. lumbricoides* IL-10 levels higher ($p < 0.0001$) in the moderately infected group. In the *A. lumbricoides* lightly parasitized children, *G. duodenalis* infection was associated to a significant increase ($p < 0.005$) of the levels of *G. duodenalis* IL-13, IFN-γ, IL-6, IgE, IgG and skin test hyper reactivity. In contrast, there was no effect of *G. duodenalis* infection in the elevation of these parameters among the *A. lumbricoides* moderately parasitized group, being those levels similarly lower as those observed in the control group. Inverse correlations were found between the levels of anti G duodenalis antibodies, skin test hyper-reactivity and cytokines with the intensity of *A. lumbricoides* infection ($p > 0.0001$) and *A. lumbricoides* IL-10 levels ($p > 0.0001$), suggesting that co-infection with *A. lumbricoides* may affect both TH1 and TH2 type immunity against *G. duodenalis* that may play an important role in the susceptibility to the infection after chemotherapy in children from endemic areas.

1. Introduction

Giardia is a ubiquitous enteric protozoan that infects humans, domestic animals and wildlife worldwide. Due to its importance in world health, *Giardia* has been included in the WHO Neglected Diseases Initiative (Savioli et al., 2006). In Venezuela, 45% of rural and 26% of urban children may be infected with *Giardia duodenalis* (Devera et al., 1998; Simoes et al., 2000).

Studies performed in animal models have indicated that components of both innate and acquired immunity are stimulated during giardiasis although the participation of these mechanisms in the elimination of the parasite has been controversial. For example, it has been demonstrated that IL-6 deficient mice are unable to control the infection compared to their wild type counterparts (Bienz et al., 2003; Zhou et al., 2003). In addition, mast cells which are an important source of IL-6 in animal experiments are crucial for controlling acute mice infections (Li et al., 2004). Mast cell degranulation leads to increases in smooth muscle contractility promoting intestinal transit and parasite elimination in mice infected with *Giardia* (Li et al., 2007). In contrast, it has been also reported that a previous and acute co-infection with *Trichinella spiralis* that induces strong TH2 type intestinal inflammation including mast cell reactivity, promotes the growth of *G. lamblia* in mice (Von Allmen et al., 2006). On the other hand, there is evidence that T cell deficient mice also fail to eliminate *Giardia* infection (Heyworth et al., 1987) and that murine macrophages treated with recombinant IFN-γ and bacterial lipopolysaccharide (LPS) ingested a significantly higher number of in vitro-grown trophozoites than untreated macrophages, indicating a possible role of IFN-γ in the clearance of the parasite (Belosevic and Daniels, 1992). Furthermore, treatment of wild-type or B-cell-deficient mice with antibodies to CD4+ T cells prevents elimination of *G. lamblia* and *Giardia muris* (Singer

* Corresponding author at: Sección de Inmunoquímica, Instituto de Biomedicina, San Nicolás a Providencia, Parroquia Altagracia, Distrito Capital., Apartado Postal: 4043, Caracas 1010 A, Venezuela. Tel.: +58 212 8615530l; fax: +58 212 8615530l.
E-mail address: isabelhagel@yahoo.com (I. Hagel).

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and Nash, 2000). Nevertheless, this study showed that the production of IFN-γ or IL-4 is not required for the protection from *G. lamblia*. Other experiments have indicated that IgA-deficient mice as well as B-cell deficient mice are not able to eradicate late infections of either *G. muris* or *G. lamblia*, suggesting that both B cells and IgA are required for the control of chronic infections (Langford et al., 2002). More recent studies showed that cyst elimination from experimental infected gerbils is associated to the presence of secretory IgA but not to serum IgG1 or IgG2 antibodies (Amorim et al., 2010). Other studies have reported that oral administration of *G. duodenalis* excretory secretory antigens in Balb/c mice is followed by an increase in the production of total and specific serum IgE (Jiménez et al., 2004) accompanied by eosinophylic infiltration, hyper-cellularity and enterocytic desquamation in the small and large intestine of the mice. These results may suggest the participation of TH2 type responses in the immunity against *Giardia* (Jiménez et al., 2004).

It has been proposed that in humans, a fully developed immune system may resolve the acute phase of the infection spontaneously emphasizing the effectiveness of innate host defenses in the control of the infection (Eckmann, 2003). Also, it has been demonstrated that human infection with *Giardia* results in increased levels of anti-*Giardia* secretory and serum IgA antibodies (Faubert, 2000; Rodríguez et al., 2004; Jiménez et al., 2009) In addition, impaired IgA responses to *Giardia* heat shock antigen have been observed in Gambian children with persistent diarrhoea and giardiasis (Char et al., 1993). Moreover, Anti-*Giardia* secretory IgA detected in human breast milk can protect lactating children against giardiasis early in life (Tellez et al., 2003). However, the participation of other possible protective mechanisms such as TH1/TH2 type cytokines and IgE or IgG antibodies in human giardiasis has been poorly studied.

On the other hand, giardiasis in tropical environments rarely occurs alone and children from endemic areas are usually co-infected with intestinal nematodes (Pennycook et al., 2000). It has been reported that chronic helminth infections may negatively influence immunity against tuberculosis and other diseases of public health importance (Markus and Finchman, 2007). Also, intestinal helminthic infections can suppress immune responses toward non-relate antigens in tropical areas where these infections are endemic (Van Riet et al., 2007). The mechanisms by which these parasites modulate other immune responses are still not clear and may vary according to the intensity of the infection (Cooper, 2009). In this work we evaluated the possible influence of *Ascaris lumbricoides* co-infection on the development of TH1 and TH2 immune responses against *G. duodenalis* as well as on the susceptibility to giardiasis in a group of Venezuelan school children chronically exposed to both parasites.

2. Methods

2.1. Study population

We carried out a longitudinal study of the school population (6–12 years) of the Madre Nueva community, formed by a small group of rural farmers (235 families) working in cacao plantations located at the coastline of San Jose de Rio Chico (10.18N; 65.59W), Miranda State, Venezuela. The community lacked adequate sanitary conditions that resulted in a high prevalence of intestinal parasitic infections (Hagel et al., 2008). A control group of healthy non-parasitized children from a private urban school from the main city of Caracas was also evaluated. The study was carried out under informed consent of the children's parents and the local health authorities from each community.

2.2. Socioeconomic and nutritional evaluation

Environmental factors such as sanitary facilities, access to running water, and poverty level, were determined according to the official Venezuelan criteria previously described (Hagel et al., 2005). Anthropometric measurements were determined according to the methods described by Gorstein (Gorstein et al., 1994). Standing height was assessed using a Harpender portable Stadiometer and weight by a DETECTO Balance (±0.1 kg). Weight/age, height/age and weight/height were determined using the WHO standard curve. Anthropometric data were analyzed with EPI INFO (Public Domain Software from Epidemiology and Disease Surveillance).

2.3. Stool examination

Three consecutive fresh stool specimens from each child were collected and examined microscopically for the presence of eggs, cysts or larvae of intestinal parasites. The Kato-Katz (Montresor et al., 1998) method was used for worm burden determinations. According to the WHO classification (Montresor et al., 1998), the intensity of *A. lumbricoides* infection was considered as light (0–5000 egg/g faeces) and moderate (5001–50,000 egg/g faeces). In Venezuela due to current ant-helminthic programs heavy worm burdens (over 50,000 egg/g feaces) are rare; therefore, the children were classified according to the presence of light or moderate *A. lumbricoides* infection and the different socioeconomic, nutritional, parasitological and immunological parameters were evaluated in each group. Due to the high risk of infection in the community under study, those children who were not parasitized at the date of evaluation were included in the lightly parasitized group. After the evaluation all the children were treated with Secnidazol (single 30 mg/kg dose). Three months after treatment the children were re-evaluated for the presence of *G. duodenalis* as well for the impact of the intensity of *A. lumbricoides* co-infection on the persistence of *G. duodenalis* infection. At the end of the study all the children attending the community school received anthelminthic treatment.

2.4. A. lumbricoides body fluid derived antigen preparation

A. lumbricoides adult vermes were collected from faeces of infected young adult volunteers from the same communities, after a 24-h treatment with Oxantel (Pfizer, Venezuela). To obtain the Ascaris body fluid (ABF) antigen, parasites were washed and preserved at −20 °C. Later, they were dissected and washed with pH 7.2 phosphate buffered saline (PBS). The parasite derived fluid obtained was partially purified by centrifugation at 12,000 × *g* (rotor SS 34 in a refrigerated Sorvall RC-5B Centrifuge) for 30 min discarding the sediment. Subsequently the extract was ultra-filtered (10,600 × *g* in a 5417R, Centrifuge, standard rotor F-45-30-11) using micro-con YM-10 filters (Millipore) for 10 min. Protein concentration was determined using the methodology described by Bradford (1976) and was found to be of 6.6 mg/ml. The homogenate obtained was aliquoted and preserved at −80 °C for subsequent trials. To each 500 μl homogenate aliquot, 10 μl of a cocktail of protease inhibitor was added (Sigma Aldrich, catalogue Not 2714).

The *G. duodenalis* antigen was obtained from in vitro axenic cultures of *Giardia* trophozoites of the P1 strain (American Type culture Collection (ATCC), No. 30888) as previously described (Rodríguez et al., 2004).

2.5. Antibody determinations

Serum levels of specific anti *G. duodenalis* IgE and IgG were measured by an ELISA assay standardized in our laboratory. Briefly, *G. duodenalis* antigen (5 μg/well) for IgE and 3 μg/well for IgG) were

coated onto 96-well micro plates (Immunolon IV, Dynatech Laboratories Inc, VA, USA) and incubated overnight at 4 °C. Excess antigen was washed off with PBS-T and plates were blocked for 2 h at 37 °C with 1% BSA. Undiluted test sera were plated and incubated for 1 h at 37 °C. After further washes with PBS-T, the plates were incubated with anti-human IgE peroxidase 1:1000 (SIGMA) or anti human IgG peroxidase 1:5000 (sigma) for 1 h. The washing process was repeated and o-phenylenediamine (OPD) plus H_2O_2 dilute in 0.05 M citrate-phosphate buffer (pH 5.5) was added. The reaction was stop using 30 μl/well of H_2SO_4 2.5 M. The O.D. was read at 490 nm. The values were expressed as Optical Density Units (ODU).

The cut–off of each assay was calculated by the mean OD units of specific antibody levels + 2 SD of 120 healthy children from a high socioeconomic private school of Caracas (0.36 ODU for IgE and 0.48 ODU for IgG). In order to compensate the variability of the values obtained in the different ELISA tests, a control pool of 20 negative samples (non-infected children) was included in each assay. The value of the pool was subtracted from each antibody measurement.

2.6. Whole blood cultures and cytokine determinations

We carried out whole blood cultures following the protocol reported by Turner (Turner et al., 2003), and adapted to our antigenic conditions. Briefly: whole blood samples of 5 ml of venous blood were collected from each child into EDTA Vacutainers (BECKTON-DICKINSON). Four ml of whole blood was diluted 1:4 with RPMI 1640 medium (SIGMA) supplemented with 2 mM L-glutamine (GIBCO) and gentamicine (80 μg/ml; GIBCO). 1 ml of the blood suspension was plated in 48-well plates and stimulated with antigen extract at a previously determined optimal concentration: 3 μg/ml of ABF antigen, 5 μg/ml of *G. duodenalis* antigen and 5 μg/ml phytohemagglutinin (PHA) (SIGMA). A fourth well was un-stimulated. All plates were incubated for 48 h at 37 °C with 5% CO_2. Supernatants were removed, centrifuged at 3000 rpm for 15 min and stored at −70 °C for future assays.

2.7. Cytokine determinations

We used the Quantikine cytokine assay (R&D Systems, Minneapolis, USA) for the determination of IL-10, IL-13, IFN-γ and IL-6 in the supernatants collected. The sensitivity of the assay was 3.9 pg/mL for IL-10, 32 pg/ml for IL-13, 8.0 pg/ml for, IFN-γ and 0.7 pg/ml for IL-6 determination. Responses to PHA and un-stimulated supernatants were recorded (data not shown) but statistical analysis was performed only in parasite-specific cytokine levels. The value corresponding to each un-stimulated well was subtracted from each respective PHA and anti-parasite cytokine measurement.

2.8. Skin prick test

Skin prick tests were carried out according to the protocol approved by the Venezuelan Clinical Society of Allergy and Immunology for clinical and research purposes (Puccio et al., 2008) using *G. duodenalis* antigen, obtained in our laboratory as previously described (Rodríguez et al., 2004), at 0.1 mg/ml in saline solution containing 50% glycerol and 0.4% phenol. Control tests were performed with the diluent alone and with 1% (w/v) histamine dihydrochloride. The wheal diameters were measured after 15 min, and diameters ≥3 mm were considered positive.

2.9. Statistical analysis

We used the Graph-Pad Instat version 3.00 for Windows 95 (GraphPad Software, San Diego California USA). Fisher's Exact Test

Table 1
Socioeconomic level, nutritional status and *G. duodenalis* infection according to the intensity of *A. lumbricoides* infection in a group of Venezuelan rural school children.

	A. lumbricoides lightly Parasitized (78)	*A. lumbricoides* moderately Parasitized (173)	Statistical significance* (relative risk)	95% Confidence interval	Urban non parasitized control group (70)	Statistical significance** (relative risk)	95% Confidence interval
Median age	9 years (6–12)	9 years (6–12)			9 years (7–12)	$p < 0.0001$ (0.677)	
Male/female	43/35	93/80			40/30		
% with access to tap water	49	57	$p = 0.2206$ (0.7905)	0.547–1.142	98		0.615–0.746
% with latrine/toilet	59	65	$p = 0.3994$ (0.846)	0.583–1.227	100	$p < 0.0001$ (0.930)	0.635–0.755
% in state of poverty	29	25	$p = 0.2611$ (0.598)	0.783–1.744	6	$p < 0.0001$ (1.276)	1.162–1.402
% Height/age <10th percentile	7	12	$p = 0.4043$ (0.629)	0.266–1.346	7	$p = 0.8109$ (1.056)	0.878–1.268
% Weight/age <10th percentile	4	6	$p = 0.759$ (0.732)	0.2248–1.762	2	$p = 0.317$ (1.198)	1.023–1.402
%Height/weight<10th percentile	4	7	$p = 0.0135$ (0.624)	0.266–2.011	3	$p = 0.382$ (1.137)	0.945–1.366
% with *G. duodenalis*	45	62	$p < 0.0001$ (0.624)	0.4316–0.904	2	$p < 0.0001$ (0.630)	0.558–0.714
% with *G. duodenalis* after treatment with Secnidazol	11	42	$p < 0.0001$ (0.268)	0.1414–0.5112	–	$p < 0.0001$ (0.604)	0.536–0.6810

* Statistical differences between *A. lumbricoides* lightly and moderately parasitized groups.
** Statistical differences between *A. lumbricoides* lightly parasitized children and the control non-parasitized group.

Table 2
Anti *G. duodenalis* specific immunological parameters according to the presence of the infection in lightly *A. lumbricoides* co-infected children.

	G. duodenalis Parasitized (45)	*G. duodenalis* Non-parasitized (33)	Statistical significance* (95% confidence interval)	Urban non-parasitized control group (70)	Statistical significance** (95% confidence interval)
Mean serum IgG (ODU)	0.831 ± 0.162	0.428 ± 0.245	$p < 0.0001$ $t = 7.132^{***}$ (−0.518 to −0.287)	0.365 ± 0.186	$p = 0.2671$ $t = 1.131^{***}$ (−0.150 to 0.176)
Mean serum IgE (O D U)	0.96 ± 0.33	0.45 ± 0.26	$p < 0.0001$ $t = 7.629^{***}$ (−0.43 to −0.376)	0.38 ± 0.22	$p = 0.1867$ $t = 1.337^{***}$ (−0.03 to 0.174)
% *G. duodenalis* s kin prick test (wheal > 3 mm)	78	22	$p < 0.0001$ Relative Risk: 3.667^{****} (1.865 to 7.209)	12	$p = 0.2339$ Relative Risk: 0.538^{****} (0.2134 to 1.361)
Supernantant IL-13 levels (pg/ml)	161.52 ± 53.68	92.35 ± 28.7	$p < 0.0001$ $t = 6.723^{***}$ (−89.66 to −48.68)	53.68 ± 21.5	$p < 0.0001$ $t = 7.621^{***}$ (−48.73 to −28.61)
Supernantant IFN-γ levels (pg/ml)	208.44 ± 56.64	22.216 ± 5.89	$p < 0.001$ $t = 9.843^{***}$ (142.6 to 229.86)	19.360 ± 6.320	$p = 0.0282$ $t = 2.243^{***}$ (−5.398 to −0.3141)
Supernantant IL-6 levels (pg/ml)	225.18 ± 45.55	113.42 ± 59.83	$p < 0.0001$ $t = 5.785^{***}$ (71.23 to 152.48)	65.5 ± 23.6	$p < 0.0001$ $t = 4.41^{***}$ (−69.8 to −26.03)
Supernantant IL-10 levels (pg/ml)	88.5 ± 47.6	87.44 ± 26.5	$p = 0.9085$ $t = 0.1154^{***}$ (−19.36 to 17.24)	66.4 ± 27.2	$p < 0.0004$ $t = 3.693^{***}$ (−32.34 to −9.73)

* Statistical differences between *G. duodenalis* parasitized and non-parasitized children.
** Statistical significance between *G. duodenalis* non-parasitized children and the control group.
*** Unpaired t Test with Welsh correction.
**** Fisher exact Test.

was used to compare positivity percentages of the different variables among the children groups. Welch's *T* test was used to compare the means of the different parameters evaluated among the different children groups. Spearman rank correlations between the distinct parameters evaluated were also performed.

3. Results

3.1. Socioeconomic and environmental factors

We evaluated a group of 251 sex-balanced school children (6–12 years) living in a rural farmer community. They all attended the same primary school and had received three meals per day at the school during the last 2 years before the evaluation. Therefore, they were well nourished and we did not find noticeable differences of the anthropometric parameters, when compared with the urban healthy control group (Table 1). All the rural children shared the same high risk from environmental factors such as lack of water supply and sanitary facilities, differing from the control urban group (Table 1).

3.2. Intestinal parasitic infection

A high prevalence of intestinal helminthes (88%) was found among the rural children, being *A. lumbricoides* (70%) and *Trichuris Trichiuria* (67%) the most frequent parasites. The prevalence of other intestinal helminthes such as hookworm and Strongyloides stercoralis was less than 12%. Also, protozoa infections were highly prevalent in this group of children: 59% of *G. duodenalis*, 33% of *Blastocistis Homminis* and 17% of *Entoamoeba histolitica/dyspar*.

We found that 31% of the rural children carried a light *A. lumbricoides* infection (0–5000 eggs/g faeces) whereas 69% of these children were moderately (5001–50,000 eggs/g faeces) parasitized. *G. duodenalis* prevalence was significantly more elevated ($p < 0.05$) among the *A. lumbricoides* moderately infected children when compared with the lightly infected group (Table 1). Three months after treatment with Secnidazol 42% of the *A. lumbricoides* moderately parasitized children were found to be infected with *G. duodenalis*, whereas only 11% of the lightly parasitized group was infected by this parasite ($p < 0.0001$) (Table 1).

3.3. Anti-G. duodenalis serum antibody levels and skin prick test positivity

In the group of *A. lumbricoides* lightly parasitized children we observed a significant elevation ($p < 0.0001$) of both specific anti *G. duodenalis* serum IgE and IgG levels among *G. duodenalis* infected children compared with their non-infected counterparts. Similar results were observed for the proportion of children with *G. duodenalis* skin test positivity (Table 2). There were no statistical differences among the *G. duodenalis* non-parasitized and the urban control group (Table 2). In contrast, those who were moderately co-infected with *A. lumbricoides* exhibited similar levels of anti *G. duodenalis* IgG and IgE antibodies as well as skin prick test positivity when compared with those observed among the non-infected children and with the control group (Table 3).

3.4. Whole blood culture-supernatant cytokine levels

A. lumbricoides-stimulated IL-10 levels were significantly elevated ($p < 0.0001$; 95% confidence interval of mean differences: −10.5 to −75.02) among the rural children when compared to the control group being extremely higher ($p < 0.0001$; 95% confidence interval of mean differences: −144.2 to −93.26) in the *A. lumbricoides* moderately parasitized children (Fig. 1).

We observed that *G. duodenalis*-stimulated cytokines profile varied according to the presence of lightly or moderately *A. lumbricoides* infection. Thus, in the lightly parasitized group, IL-13, IFN-γ and IL-6 levels were extremely higher ($p < 0.0001$) among the *G. duodenalis* parasitized children when compared with their non-infected counterparts. Also, there were significant statistical differences between *G. duodenalis* non-parasitized children and the control group (Table 2). In contrast, there were no statistical differences in these parameters between *G. duodenalis* parasitized and

Table 3
Anti *G. duodenalis* specific immune parameters according to the presence of *G. duodenalis* in moderately *A. lumbricoides* co-infected children.

	G. duodenalis Parasitized (104)	*G. duodenalis* Non-parasitized (69)	Statistical significance* (95% confidence interval)	Urban non-parasitized control group (70)	Statistical significance** (95% confidence interval)
Mean serum IgG (ODU)	0.361 ± 0.126	0.326 ± 0.140	$p = 0.0889$ $t = 1.711^{***}$ (−0.075 to 0.053)	0.365 ± 0.186	$p = 0.1652$ $t = 1.395^{***}$ (−0.094 to 0.016)
Mean serum IgE (O D U)	0.47 ± 0.15	0.43 ± 0.17	$p = 0.1054$ $t = 1.628^{***}$ (−0.088 to 0.0085)	0.38 ± 0.22	$p = 0.1365$ $t = 1.498^{***}$ (−0.016 to 0.116)
% skin prick test (wheal > 3 mm	19	16	$p = 0.686$ Relative Risk: 0.867^{****} (0.450 to 1.670)	12	$p = 0.3454$ Relative Risk: 0.643^{****} (0.285 to 1.452)
Supernantant IL-13 levels (pg/ml)	84.47 ± 35.14	94.6 ± 36.34	$p = 0.0688$ $t = 1.832^{***}$ (−0.787 to 21.4)	65.8 ± 27.2	$p < 0.0002$ $t = 3.751^{***}$ (−28.49 to −8.847)
Supernantant IFN-γ levels (pg/ml)	22.054 ± 9.64	21.32 ± 6.05	$p = 0.5923$ $t = 0.5378^{***}$ (−3.438 to 1.970)	19.360 ± 6.320	$p = 0.0640$ $t = 1.867^{***}$ (−0.1156 to 4.036)
Supernantant IL-6 levels (pg/ml)	69.26 ± 24.18	68.32 ± 14.26	$p = 0.7715$ $t = 0.2909^{***}$ (−7.318 to 5.438)	65.5 ± 23.6	$p = 0.3962$ $t = 0.8511^{***}$ (−3.732 to 9.372)
Supernantant IL-10 levels (pg/ml)	80.92 ± 27.4	91.54 ± 27.35	$p = 0.8842$ $t = 0.1458^{***}$ (−7.72 to 9.012)	62.5 ± 21.3	$p < 0.0001$ $t = 4.583^{***}$ (−27.25 to −10.82)

* Statistical differences between *G duodenalis* parasitized and non-parasitized children.
** Statistical significance between *G. duodenalis* non-parasitized children and the control group.
*** Unpaired *t* test with Welsh correction.
**** Fisher exact test.

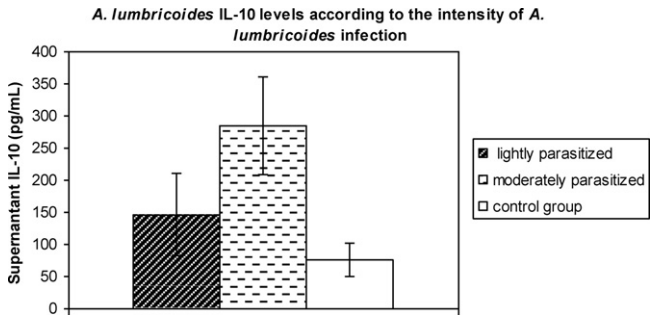

Fig. 1. *A. lumbricoides* IL-10 levels according to the intensity of *A. lumbricoides* infection.

non-parasitized groups in those children who were co-infected with a moderate *A. lumbricoides* worm burden (Table 3). *G. duodenalis* stimulated IL-10 levels were significantly elevated among *G. duodenalis* non-parasitized children when compared with the control group (Tables 2 and 3) but there were no statistical significances according to presence of *G. duodenalis* infection among the rural groups.

We found significant inverse correlations between anti *G. duodenalis* serum IgG, IgE levels as well as stimulated IL-13, IFN-γ and IL-6 levels with the intensity of *A. lumbricoides* infection and with *A. lumbricoides* stimulated IL-10 levels (Table 4). The same pattern was observed for skin test hyper-reactivity. In addition, a significant correlation was found between *A. lumbricoides* IL-10 levels and the proportion of *G. duodenalis* infected children after 3 months treatment with Secnidazol ($p < 0.0001$).

4. Discussion

Giardia is a worldwide parasitic infection that causes intestinal symptoms ranging from diarrhoea to constipation, nausea, headache and flatulence (Flanagan, 1992), affecting the quality of life of many children living at conditions of poverty which place them more susceptible to intestinal infections (Savioli et al., 2006). The understanding of the mechanisms involved in the immunity against *G. duodenalis* may contribute to the design of strategies to control or eliminate this parasitic infection. However, little is known about these mechanisms in human populations. On the other hand, individuals living in rural endemic areas are usually co-infected by more than one intestinal parasite which may compromise the immune response against a particular intestinal pathogen (Soboslay et al., 2006). Our results indicated that *G. duodenalis* infection can stimulate the production of specific TH1 and TH2 type cytokines and antibodies nevertheless these responses may be modulated according to the intensity of *A. lumbricoides* co-infection.

Therefore, we observed that lightly *A. lumbricoides* co-infected children showed elevated levels of stimulated-IL-13, IL-6, IFN-γ cytokines as well as serum IgE and IgG antibodies against *G. duodenalis* antigens compared to their non-infected counterparts. Previous studies carried out in children parasitized with *G. duodenalis* have shown an elevation of a mixed TH1/TH2 pattern of cytokines (Matowicka-Karna. et al., 2009) as well as of anti-parasite IgG, IgA and IgE serum antibody levels (Jiménez et al., 2009). Also, in vitro studies have indicated that inflammation at the gastric mucosa of *G. intestinalis*-infected children is characterized by local production of cytokines such as IL-5, IL-4, IL-6 and IFN-γ (Maciorkowska et al., 2005). The stimulation of IL-13 by *Giardia* derived antigens has not been previously reported. This cytokine promotes IgE class switching (Mc Kenzie et al., 1998; Zitnik et al., 2009) and thus may be associated to the high levels of serum IgE and skin prick test positivity toward *G. duodenalis* antigens observed among these children. The elevation of allergic reactivity that have been also previously reported in children infected with *G. duodenalis* (Di Prisco et al., 1998; Mahmoud et al., 2004; Melo-Reis et al., 2007) may reflect mast cell activity stimulated by parasite antigens. Although, the possible role of intestinal mast cells in human giardiasis needs further investigations.

In contrast to our observations among the lightly parasitized group of children, moderate *A. lumbricoides* loads were negatively associated with the levels of serum anti-*G. duodenalis*

Table 4
Spearman rank correlation between different immunological parameters in children co-infected with *A. lumbricoides* and *G. duodenalis*.

N = 251	Serum Anti *G duodenalis* IgG (ODU)	Serum Anti *G duodenalis* IgE (ODU)	Anti *G. duodenalis* Skin test Hyper-reactivity	*G. duodenalis* IL-13 (pg/ml)	*G. duodenalis* IFN-γ (pg/ml)	*G. duodenalis* IL-6 (pg/ml)	% *G. duodenalis*-infected children after treatment with Secnidazol
A. lumbricoides IL-10 (pg/ml)	$r = -0.233$ $p = 0.0327$	$r = -0.0335$ $p = 0, 0018$	$r = -0.5283$ $p < 0.0001$	$r = -0.3091$ $p = 0.042$	$r = -0.5168$ $p < 0.0001$	$r = -0.4034$ $p < 0.0001$	$r = 0.5703$ $p < 0.0001$
A. lumbricoides Worm burden (eggs/g feaces)	$r = -0.4394$ $p < 0.0001$	$r = -0.3917$ $p = 0.0002$	$r = -0.432$ $p < 0.0001$	$r = -0.2711$ $p = 0.013$	$r = -0.598$ $p < 0.0001$	$r = -0.733$ $p < 0.0001$	$r = 0.4710$ $p < 0.0001$

antibody responses as well as with the in vitro stimulation of pro-inflammatory cytokines by *G. duodenalis* antigens. We found that the decrease in these parameters was associated to a strong elevation of IL-10 levels stimulated by *A. lumbricoides* antigens. In addition, other studies have demonstrated that African children under conditions of hyper endemic exposure to *A. lumbricoides* and *T. trichiura*, constitutively secrete more immunoregulatory cytokines such as IL-10 and transforming growth factor (TGF)-beta1 compared with those observed in children under conditions of meso-endemic exposure. The production of these cytokines correlated negatively with the levels of IL-4, IFN-γ as well as with cellular proliferative responses to *A. lumbricoides* or *T. trichiura* helminth antigens, Streptococcus pneumoniae bacterial antigen and the mitogen phytohemaglutinin (Turner et al., 2008). There is evidence that nematode derived molecules such as cysteine proteases can stimulate the production of high IL-10 levels in human blood mononuclear cell cultures (PBMC) (Hartmann et al., 1997). This cytokine is involved in the down regulation of the expression of co stimulatory molecules such as CD86 and HLA-DR expression, interfering with the antigen presentation process (Hartmann and Lucius, 2003) and consequently with the development of both TH1 and TH2 immune responses. It has been proposed that these mechanisms appeared as a stratagem to modulate host immune responses against the parasite, thus maintaining a beneficial environment which allows them to survive and avoid harmful inflammatory processes in the host (Van Riet et al., 2007; Soares and Araujo, 2008). However, this activity may spread suppression to non-cognate antigens interfering with the immune response to other infectious agents.

The possible protective role of TH1 or TH2 type immunity in *Giardia* infection is still unclear. As mentioned above evidence from experimental models has been controversial. In humans yet there are very few reports available. For example, it has been shown that *Giardia* can stimulate the production of IFN-y from human CD4+ T cells in vitro, although they do not trigger cytotoxicity or migration (Ebert, 1999). A more recent study has shown that elevated levels of IFN-γ, IL-4, and IL-5 cytokines in feacal samples from infected individuals are associated with an increase in the duration of *G. lamblia* infection suggesting that these cytokines may favor the persistence to the infection (Long et al., 2010). Nevertheless, our results indicated that the capacity to stimulate TH1 and TH2 type cytokines and antibodies against *G. duodenalis* was associated to a reduction in the proportion of infected individuals before and after anti-protozoa treatment. Moreover, suppression of these responses in the *A. lumbricoides* moderately infected group placed these children to a greater susceptibility to the infection than their lightly infected counterparts. Therefore, more studies are needed to elucidate the role of these mechanisms in the protection against *G. duodenalis*. Besides, other components of the immune response which were not considered in this study could also be influenced by *A. lumbricoides* co-infection, thus contributing to the susceptibility to *G. duodenalis* in these children. Anyhow, the capacity of *A. lumbricoides* to modulate the immune response against other intestinal pathogens may constitute an important factor to take into account in the design of future control programs in endemic areas.

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