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Role of the low affinity IgE receptor (CD23) on the IgE response against *Ascaris lumbricoides* in Warao Amerindian children from Venezuela.

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Key words: Warao Amerindian children, *A. lumbricoides,* IgE, CD20+CD23+ cells.

Abstract. The objective of this study was to investigate the immune mechanisms against gastrointestinal helminths involved in the production of IgE, particularly the role of the low affinity IgE receptor (CD23) in Amerindian Warao children from the Orinoco Delta, Venezuela. We studied a group of unselected (n = 159) Warao school children with high prevalence and intensity of helminthic infection and a similar non- parasitized (n = 70) control group of a "creole" community also located at the Delta of the Orinoco. The levels of total and specific anti-*Ascaris* IgE, determined by ELISA, were extremely higher in the Warao children ($p < 0.001$) compared to the control group. However, only a reduced group of these children were able to develop a clinically significant (> 0.7 PRU/mL) IgE response against *A lumbricoides* antigens. Circulating T helper cells (CD3+CD4+), activated B (CD20+, CD20+CD21+) lymphocytes and the expression of the low affinity receptor for IgE (CD23), determined by flow cytometry, were significantly ($p < 0.001$) elevated in the Warao children, particularly in those with an enhanced capacity to mount efficient specific anti *Ascaris* IgE responses. Strong correlations ($p < 0.001$) were found between the number of circulating CD20+CD23+ cells and the level of total and anti-*A lumbricoides* IgE. Also, a significant ($p < 0.005$) correlation between CD20+CD23+ and CD4+ circulating cells was observed. We conclude that *A lumbricoides* antigens may stimulate the production of total and specific IgE through a CD23 dependent pathway. However the different environmental and genetics factors involved in the capacity to develop efficient specific responses among the Warao population still need to be elucidated.

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Papel del receptor de baja afinidad para la IgE (CD23) en la respuesta IgE frente a *Ascaris lumbricoides* en niños indígenas Warao de Venezuela.

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Palabras clave: Niños indígenas Warao, *Ascaris lumbricoides*, IgE, células B CD20+CD23+.

Resumen. El objetivo de este estudio fue investigar los mecanismos inmunitarios frente a helmintos gastrointestinales involucrados en la producción de IgE, particularmente el papel del receptor de baja afinidad para la IgE (CD23) en niños indígenas de la etnia Warao del Delta del Orinoco en Venezuela. Estudiamos un grupo no seleccionado (159) de niños Warao escolares con una alta prevalencia e intensidad de infección por parásitos helmintos, y un grupo control de 70 niños no parasitados de una comunidad rural criolla del mismo Estado. Los niveles totales y específicos de IgE anti-*A lumbricoides*, determinados por ELISA, fueron extremadamente elevados en los niños Warao ($p < 0,001$). Sin embargo, solo un reducido grupo de esos niños fue capaz de desarrollar una respuesta de IgE específica clínicamente significativa (> 0,7UI/mL) frente a antígenos de *A lumbricoides*. El número de células circulantes, T CD4+, B CD20+ CD21+ y la expresión del receptor de baja afinidad para IgE (CD23) determinados por citometría de flujo, fueron significativamente ($p < 0,001$) más elevados en los niños Warao, particularmente en aquellos con adecuados niveles de IgE específica frente al parásito. Se encontró una alta correlación ($p < 0,001$) entre los niveles totales y específicos de IgE específica y la expresión del receptor para la IgE (CD23) y una correlación significativa ($p < 0,005$) entre el número de células circulantes CD20+CD23+ y CD4+. Concluimos entonces que los antígenos de *A. lumbricoides* podrían estimular la producción de IgE total y específica a través de una vía dependiente del receptor CD23. Sin embargo, los factores ambientales y genéticos que podrían determinar la capacidad de desarrollar respuestas IgE eficientes en la población Warao, requiere de mayor estudio.

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INTRODUCTION

The Amerindian Warao ethnic group constitutes a population of around 21000 people dispersed into 250 isolated fishing and hunting communities along the delta of the Orinoco River, in eastern Venezuela. They live in extreme poverty with very poor sanitary conditions and limited access to health assistance, which place the children at high biomedical risk (1). Our previous studies in these communities have shown a high prevalence of helminthic infection (75%), particularly *Ascaris lumbricoides* (62%), as well as elevated total (8500 IU/mL) and specific anti-*A lumbricoides* (3.5 PRU/mL) IgE serum levels (2). It has been extensively reported that the stimulation of specific IgE antibodies by helminth antigens constitutes an important compo-

nent of the protective response against these parasites (3, 4). Our previous investigations in children from slum areas, with a high prevalence of helminthic infection, have shown that children with the highest specific IgE levels against adult worm antigens are less likely to be re-infected with *A. lumbricoides* after antihelminthic treatment (5). In addition, children with an atopic background that have demonstrated an enhanced capacity to produce high levels of specific IgE antibodies against these parasites, had significantly lower intensities of infection than their non atopic counterparts (6).

IgE switching is dependent on two signals provided by specifically stimulated T cells: IL-4 induces the expression of the sterile transcript, and the triggering of CD40 with its ligand induces the expression of the mature transcript coding for IgE (7). It has been proposed that additional signals are also required for IgE production; these would be provided by different cytokines, soluble CD23 and the pairing of B cell surface molecules CD21 and CD23 (8). Antibodies to CD23 have been shown to inhibit IL-4 induced human IgE production *in vitro* (9) and to inhibit antigen -specific IgE responses in rat models in an isotype-selective manner (10). Studies performed in human populations have reported an increase in the proportion of CD23+ circulating B cells in asthmatic children allergic to *Dermatophagoides pteronyssinus*, with high levels of specific IgE against this allergen (11).

The aim of this study was to evaluate the possible associations between serum levels of total and specific IgE against *Ascaris lumbricoides* and the low affinity IgE receptor (CD23) expression on B lymphocytes circulating cells in a group of Amerindian Warao school-children chronically exposed to gastrointestinal helminths.

SUBJECTS AND METHODS

Study group

We performed a cross sectional study in a group of 159 school children from a Warao Amerindian community, Arawuaymujo, located at the delta of the Orinoco River. The population studied was sex balanced and the age range of the group (6-11 years old; mean age: 8.6 2.5 years) was selected according to previous studies in which the prevalence and intensity of helminthic infection, have shown to be comparable at this age range (12). A control group of 70 healthy non parasitized school-children (mean age 7.6 2.5 years) was also evaluated. These children belong to the community of El Caigual, also located near the Orinoco river at Delta Amacuro state. Ethnically, this community belongs to the "creole" population. However, most of these children may have a Warao ancestor. Socioeconomic aspects that may influence the prevalence and intensity of helminthic infection, including sanitary conditions, mother's level of education and the degree of poverty were determined in both groups of children (Table I). The degree of poverty of the families was determined by applying the criteria in official use in Venezuela, as defined by the Central Bank of Venezuela and the Central Office of the Co-ordination of the President of the Bolivarian Republic of Venezuela (CORDIPLAN) (12). According to this classification, the total family incomes are compared to the cost of a "shoping basket" of basic food items, and the cost of basic food and services for a standard family. Accessing to alimentary practices like fishing or hunting was also considered in the study of these communities. Those families whose incomes did not cover the basket of basic food items and had limited access to other alimentary practices were considered as being in extreme poverty.

TABLA I
SOCIOECONOMIC CONDITIONS OF THE DIFFERENT GROUPS OF CHILDREN EVALUATED

% of children	Araguaymujo (159)	El Caigual (70)	Statistical significance	95% confidence interval
Without access to tap water	95	15	P < 0,0001 Odds ratio: 101,24	38,788 to 264,24
Lack of toilet or latrine	80	16	P < 0,0001 Odds ratio:21,287	10,039 to 45,137
Illiterate mother	12	15	P = 0,5239 Odds ratio: 0,7279	0,3262 to 1,624
Extreme poverty (according to their family income)	35	28	P = 0,3630 Odds ratio: 1,359	0,7368 to 2,507

Ethical considerations

Informed parental consent was obtained to participate in the study. This project was approved by the ethical committees of the Regional Department of the Ministry of Health at Delta Amacuro State, and of the Institute of Biomedicine. The study was also approved by the National Council for Scientific and Technological Investigation of Venezuela, and the Postgraduate Commission of the Faculty of Medicine of the Central University of Venezuela. During the study, medical assistance was provided to all the people living in the community and children were treated for helminthic infections and other common childhood diseases.

Parasitologic evaluation

Faeces samples were collected and direct examination of the samples was performed. The helminth intensities were determined by the method of Kato (14). *Strongyloides* intensity was assessed by the Baermann concentration technique (14). The number of helminth eggs was calculated per gram of faeces (epg) in all the samples.

Total serum IgE levels

These were measured by a capture ELISA developed in our laboratory and standardized against the commercial Phadebas IgE PRIST (Pharmacia Sweden) test. Briefly, flat-bottom 96-well microtitre plates (Immunolon IV Dynatech Laboratories Inc, Virginia USA) were coated overnight at 4°C with 1μg/well of murine monoclonal anti-human IgE (produced in the Institute for Child Research, Perth Australia) in carbonated buffer (pH 9.6) and blocked with 1% bovine serum albumin (BSA). The plates were washed with phosphate buffer saline containing 0.1% Tween 20 (PBS-T, pH 7.4) then incubated with serum samples diluted 1:400 for 2 hours at 37°C. After another wash, biotinylated murine anti-human IgE diluted 1:500 (Vector laboratories, California, USA) was added and incubated for 1 hour at 37°C. The plates were washed thoroughly and allowed to react with Avidin-Biotin-Peroxidase complex (Vectastain kit, Vector laboratories) for 30 minutes at 37°C. -phenylenediamine (OPD; SIGMA, USA) plus H_2O_2 were added, and the O.D. was read at 490 nm. A standard titration curve using a pool of sera with high IgE levels, according to the

Phadebas IgE PRIST, was run with each assay. Results were expressed as International units/mL (IU/mL).

Anti-Ascaris lumbricoides IgE levels

These were also measured by an ELISA developed in our Laboratory and standardized against the commercial RAST technique. *A. lumbricoides* adult worm antigen (15), 3μg/well, was coated onto 96-well microplates (Immunolon IV, Dynatech Laboratories Inc, Virginia USA) and incubated overnight at 4°C. Excess antigen was washed off with PBS-T and the plates were blocked for two hours at 37°C with 1% BSA. The test undiluted sera, were plated and incubated for 1 hour at 37°C. After further washes with PBS-T, the plates were incubated with 1μg/well of murine monoclonal anti-human IgE (produced in the Institute for Child Research, Perth, Australia) for 1 hour and then washed off with PBS-T, then the plates were incubated with peroxidase -conjugated anti-mouse IgG diluted 1:3000 (Sigma, USA). The washing process was repeated and o-phenylenediamine (OPD) plus H_2O_2 were added. The optical densities were read at 490nm. A standard titration curve using a pool of sera with high specific anti-*Ascaris* IgE levels in the commercial Phadebas test was run with each assay, and the results were expressed as Phadebas RAST units (PRU/mL).

Lymphocyte surface markers

Peripheral blood samples were obtained by venipuncture, and collected into EDTA anticoagulant. Routine haematological studies were performed in the clinical laboratory of the nearest Hospital (Tucupita, Delta Amacuro State), and flow cytometry assays were undertaken at the Municipal Blood Bank of Caracas, using a FACScan Flow Cytometer (Becton Dickinson, San Jose, California, USA). Dual colour (FITC-PE) immunofluorescent staining of cell surface antigens in unseparated blood was performed using monoclonal antibodies for the detection of the following celular phenotypes: T helper cells, CD3+CD4+, (FITC/PE), activated B cells, CD20+CD21+, (PE/FITC), and B cells expressing the low affinity receptor for IgE, CD20+CD23+, (FITC/PE). The monoclonal antibodies used were purchased by DAKO, Denmark. 10.000 cells were acquired for each analysis performed. The results were expressed as absolute number of lymphocytes/mm^3.

Statistical Analysis

The total and specific IgE levels and the eggs per gram of faeces were logarithmically transformed. Geometric mean and geometric mean + 1 standard deviation were calculated for these parameters. A parametric analysis was performed. Students' t test and Welch t test were used for comparing means of the different parameters between the groups. Comparison between the proportions of children was accessed by the Fisher exact test. The Pearson rank correlation coefficient was used to determine the significance of the different correlations calculated.

RESULTS

We found that the degree of poverty, according to family income, was comparable between the two communities evaluated. However, extremely poor sanitary conditions were found among the Warao children from Arawaymujo, with an elevated prevalence and intensity of gastrointestinal helminthic infection. In contrast, improved sanitary facilities and adequate access to primary health care were found among the non-parasitized group from El Caigual (Table I). Despite the high total serum IgE levels observed in the parasitized group (7630 UI/mL), only 32.7% of these children exhibited clinically significant (0.7 PRU/mL) specific anti-*Ascaris*

IgE levels (geometric mean: 4.5 PRU/mL) according to the international RAST Classification (PHARMACIA, Sweden). These were classified as "high specific anti-*Ascaris* IgE responders". The others had specific anti-IgE levels under 0.7 PRU/mL (geometric mean: 0.36 PRU/mL) and were considered as "low specific-anti-*Ascaris* IgE -responders". Geometric mean of *Ascaris* specific IgE among the non-parasitized group was 0.46 PRU/mL.

Table II shows there was no significant difference in the prevalence of helminthic infection between high specific anti-*Ascaris* IgE responders and their low responder counterparts. The prevalence and intensity of *Strongyloides* infection was very low in all the children and was considered as sub-clinical.

The intensity of *Ascaris* and *Trichuris* infection was significantly lower ($p = 0.0002$ and $p < 0.0001$ respectively) in the high specific anti-*Ascaris* IgE responder group (Table III). Total serum IgE levels were extremely higher in the Warao children compared to the non-parasitized control group ($p < 0.001$), and they were also higher ($p = 0.00637$) in the high specific anti -IgE responder group (Table III).

We also found that the absolute number of peripheral circulating T cells (CD3+, CD3+CD4+) was significantly higher ($p < 0.0001$) in the Warao children compared to the non-parasitized control group; the circulating T helper (CD3+CD4+) cell sub-population was significantly higher ($p < 0.0001$) in the "high specific anti-IgE responder" children compared to the low responder group. Similarly, B lymphocyte circulating sub-populations (CD20+, CD20+CD21+ and CD20+CD23+) were significantly higher ($p < 0.001$) in the Warao children (Table IV).

The expression of the low affinity IgE receptor (CD23) was significantly higher ($p < 0.0001$) in the high responder group (Table IV). Significant correlations (Pearson rank: 0.6450; $p < 0.001$) between CD23 ex-

TABLE II
HELMINTHIC INFECTION IN A GROUP OF WARAO AMERINDIAN CHILDREN ACCORDING TO THEIR SPECIFIC ANTI-*Ascaris* IgE LEVELS

	High specific Anti-*Ascaris* IgE Responders 0.7IU/mL (52)	Low specific anti-*Ascaris* IgE Responders < 0.7IU/mL (107)	Statistical significance	95% confidence interval	Non-parasitized control group (70)
Prevalence of total helminthes (%)	68	72	P = 0.5811 Odds ratio: 0.8021	0.3917 to 1.643	-
Prevalence of *Ascaris lumbricoides*	54	59	P = 0.6095 Odds ratio: 0.8148	0.4179 to 1.589	-
Prevalence of *Trichuris trichuria*	45	53	P = 0.6957 Odds ratio: 0.695	0.3573 to 1.355	-
Prevalence of *Strongyloides stercoralis*	12	18	P = 0.3614 Odds ratio: 0.6041	0.2256 to 1.618	-

TABLE III

TOTAL SERUM IgE LEVELS IN A GROUP OF WARAO AMERINDIAN CHILDREN ACCORDING TO THEIR SPECIFIC ANTI-*Ascaris* IgE LEVELS

	High specific anti-*Ascaris* IgE Responders 0.7IU/mL (52)	Low specific anti-*Ascaris* IgE Responders < 0.7IU/mL (107)	Statistical significance	95% confidence interval of the difference	Non-parasitized control group (70)
Geometric mean of No of *Ascaris* eggs /gram faeces (Geometric mean +1SD)	3214 (8500)	5550 (12300)	P = 0.0002 t: 3.768	0.1129 to 0.3615	-
Geometric mean of Nº of *Trichuris* eggs /gram faeces (Geometric mean +1SD)	1345 (3450)	2225 (3850)	P < 0.0001 t: 4.249	0.1170 to 0.3202	-
Geometric mean of total serum IgE levels (Geometric mean +1 sd)	8300 (12650)	6450 (16300)	P = 0.00637 t: 1.867	–0.2253 to 0.00631	450** (1100)

** P < 0.0001. t 18.946. 95% confidence interval: 1.036 to 1.277. Between Low specific anti-*Ascaris* IgE responders and non-parasitized control group.

TABLE IV

ABSOLUTE NUMBER OF PERIPHERAL CIRCULATING LYMPHOCYTES IN A GROUP OF WARAO AMERINDIAN CHILDREN ACCORDING TO THEIR SPECIFIC ANTI-*Ascaris* IgE LEVELS

Lymphocytes/ mm^3	Responders 0.7PRU/mL (52)		Non-responders < 0.7PRU/mL (107)		Statistical significance	95% confidence interval of the difference	Non Warao Non Infected control group (70)		Statistical significance	95% confidence interval
CD3+	2617.3	602.7	2169.1	499.2	P < 0.0001 t: 4.956	–626.84 to –269.56	1580.2	342.5	P < 0.0001 t: 8.627	–723.63 to –454.17
CD3+CD4+	1577.3	368.4	1166.8	295.61	P < 0.0001 t: 7.563	–517.70 to –3.03	780.3	126.4	P < 0,0001 t: 23.695	–9060.34 to –812.66
CD20+	636.2	285.3	618.1	236.08	P = 0.6729 t: 4.230	–102.62 to 6.416	480.5	126.3	P < 0.0001 t: 4.473	–198.32 to –76.88
CD20+CD21+	536.9	253.5	525.4	100.2	P = 0.7536 t: 0.3154	–84.489 to –61.489	320.5	130.5	P < 0.0001 t: 11.783	–239.22 to 170.58
CD20+CD23+	334.9	125.7	159.4	69.8	P < 0.0001 t: 9.387	–212.82 to –138.18	95.2	46.4	P < 0.0001 t: 6.807	–82.813 to –45.587

pression and total serum IgE levels and also with specific anti *Ascaris* IgE levels (Pearson rank: 0.573; $p < 0.05$) were observed. Moreover, the number of circulating CD20+ CD23+ cells correlated strongly (Pearson rank: 0. 6870; $p < 0.001$) with the number of circulating T helper cells (CD3+CD4+).

DISCUSSION

The Warao people constitute the most numerous and ethnically conserved Amerindian population in Venezuela, despite the hostile environmental conditions that surround them (1). Probably, the development of adaptive defence mechanisms against infectious diseases could play an important role in their survival (16).

As expected, gastrointestinal helminths were highly prevalent among children from this Warao community living in a sanitary deprived environment, *Ascaris lumbricoides* being the most frequent. In agreement with this situation, extremely elevated levels of total serum IgE were observed among this group of children. Independently of their extremely high total IgE levels, the majority of the Warao children studied were found to produce scarcely detectable levels of specific anti-*Ascaris* IgE; only a reduced group of children were capable of mounting a strong effective specific response against this parasite. The prevalence of helminthic infection was similar between both high and low IgE responder groups, indicating a comparable risk of exposure to these parasites. However the intensity of *Ascaris* infection was significantly lower in the "high specific anti *Ascaris* IgE responder" group. This is in agreement with our previous findings, where atopic children from rural areas with an enhanced capacity to produce specific IgE antibodies against these parasites, had significantly lower intensities of infection than their non atopic counterparts (6), and suggests that the capacity to establish efficient specific IgE responses may be an advantage that confers better defence mechanisms against helminthic infections.

In addition, and probably as a reflection of the chronic stimulation of the immune system by continuous infectious diseases, particularly those caused by gastrointestinal parasites, a significant increase in the number of circulating T helper (CD3+ CD4+) and activated B cell (CD20+, CD20+CD21+) sub-populations was also found among these children compared to the non-parasitized control group. Correspondent to the elevation in the levels of total and specific anti *Ascaris* serum IgE, the expression of the low affinity IgE receptor (CD20+ CD23+) was markedly augmented in the Warao children, being extremely high in the "high specific anti- *Ascaris* IgE responder" group. Moreover, a strong correlation between the number of CD23+ B cells and both total and specific anti *Ascaris* serum IgE levels was also observed. The mechanisms by which helminth parasites may enhance CD23 expression are still not well elucidated. Experimental evidence obtained from *in vitro* assays have demonstrated that excretory-secretory components of filarial parasites are capable of stimulating the TH2 derived, IL-4-dependent expression of CD23 by human splenic B cells (17). Other authors have proposed that helminth proteins with anti-protease activities may affect the processing of B cell surface molecules involved in IgE switching, particularly CD23, and consequently the regulation of IgE synthesis (18). In this study, we found a significant correlation between the number of CD23+ B and CD4+T helper circulating cells, indicating that antigens derived from gastrointestinal

helminths, such as *Ascaris lumbricoides,* may stimulate the expression of the low affinity IgE receptor (CD23) in a CD4+ dependent manner.

Studies using inhibitory anti CD21 antibodies have shown that CD23 binds to a subtype of CD21 (8), a molecule that has previously identified as the complement receptor 2 and as an activator B cell factor (19). Moreover, it has been demonstrated that triggering CD21 by recombinant sCD23 enhances IgE production in both T cell dependent and T cell independent systems (20). Thus, it is possible that in children populations living in deprived sanitary environments, at a situation of high biomedical risk, early expression of CD21 receptor may occur, favouring the pairing of CD21/CD23 positive B cells in parasitized populations, therefore, enhancing the production of high levels of total and specific IgE.

However, other factors may also be involved in the generation of protective specific anti-*Ascaris* IgE responses. The capacity to recognize specific epitopes that may confer protection against helminths (21) in these Warao children must be identified. Other aspects, such as malnutrition, that affect the capacity to develop efficient memory T helper responses (22) have been shown to be important for the stimulation of high affinity specific IgE responses against these parasites (23). On the other hand, recent work performed by Turner et al, has demonstrated a positive association between blocking IgG4 antibodies against *Ascaris* antigens and the intensity of *Ascaris lumbricoides* infection in chronically exposed children (24). Therefore, the complex interactions of different factors that may determine the capacity to establish protective IgE responses against these parasites, among these groups of children, need further investigation.

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