Secretory IgA Antibody Responses in Venezuelan Children Infected with *Giardia duodenalis*

by Orquídea L. Rodríguez, Isabel Hagel, Yovana González, María Elena Roque, Nelson Vásquez, Elianska López, *and* María Cristina Di Prisco

Institute of Biomedicine, Faculty of Medicine, Central University of Venezuela, Venezuela

Summary

We standardized and evaluated an ELISA technique for the detection of total and specific anti-*Giardia duodenalis* secretory IgA antibodies (sIgA). Samples of saliva and serum of 161 Venezuelan schoolchildren were analysed. After stool examination, 66 children were diagnosed to be infected with *Giardia duodenalis*, 22 with other protozoa, and 73 non-parasitized. The mean (+ 2 SD) values of secretory IgA in the non-parasitized group was considered as the criterion of positivity. The levels of total and specific anti-*Giardia* sIgA were significantly higher in children with *Giardia* compared with the group with other protozoa ($p < 0.01$) and the non-parasitized group ($p < 0.001$). The ELISA technique developed showed values of sensitivity and specificity of 74 and 94 per cent, respectively, a predictive value of 92 per cent for positive samples and 80 per cent for negative samples. Specific anti-*Giardia* IgA serum levels showed a low sensitivity (57 per cent) and a predictive value for negative samples (53 per cent). Our results suggest that secretory anti-*Giardia* IgA levels measured in saliva samples may reflect local intestinal IgA responses elicited by these parasites. Thus, determinations of the levels of sIgA anti-*Giardia* could be a useful diagnostic tool for giardiasis in children.

Introduction

Giardiasis is a gastrointestinal infection of wide distribution, more prevalent in childhood.[1,2] Prevalence of 20–60 per cent has been reported in some areas of developing countries.[3] In Venezuela, studying human groups from different regions and socioeconomic levels, we found an increasing prevalence according to the descending socioeconomic level of the populations with a range between 20 and 40 per cent.[4,5] Therefore, it constitutes an important public health problem in our country. An association between active infection and stunting has been demonstrated in children infected with *Giardia*, and eradication of the infection with appropriate treatment often produces the disappearance of the symptoms and an improvement in growth;[6] thus, early diagnosis seems to be important. Conventional diagnosis by direct microscopic examination of single or serially collected stool samples, to determine the presence of parasite cysts or trophozoites, has been shown to be of moderate sensitivity due to intermittent shedding of parasite cysts and trophozoites in the stool.[7,8] Alternative methods of diagnosis, such as duodenal context examination or intestinal biopsies, are more sensitive, but they are considered to be invasive and risky procedures, especially in children. Diverse serological methods[9,10] and solid-phase immunoenzymatic assays for the detection of parasite antigens have been developed.[11,12] It has been reported that circulating specific IgG antibodies can persist for a long period after infection and can also be detected in newly infected people. However, they cannot be used to differentiate among acute, chronic, or past infections. Even though the determination of IgM antibodies may be useful to discern the state of the infection,[10] other authors have reported that to obtain better results the IgM and IgG antibodies should be previously separated by chromatography and then quantified, making the procedure much more cumbersome and prolonged.

Secretory IgA (sIgA) is the predominant antibody in the intestinal lumen and probably the most important mechanism involved in defence mechanisms against this parasite.[13] It has also been reported that people with IgA deficiencies are more susceptible to infection by *Giardia duodenalis*.[14] Specific anti-*Giardia* sIgA has been found in duodenal juice samples of infected patients using ELISA and also on the surface of *Giardia* trophozoites from biopsies of human jejunum by direct immunofluorescence techniques,[15] strongly suggesting the production of this antibody during active *Giardia* infection. Therefore,

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Correspondence: Tel. +58-212-860 70 95; Fax +58-212-861 55 30. E-mail <orquileo@hotmail.com; ihagel@telcel.net.ve>.

the determination of secretory IgA may be an important tool for monitoring the course of the infection.

The aim of this investigation was to study the IgA response in children with giardiasis diagnosed by the finding of cysts or trophozoites in stool samples, and to develop and evaluate an immunoenzymatic solid phase (ELISA) assay for the detection of total secretory IgA and specific serum and secretory anti-*G. duodenalis* IgA antibodies.

Materials and Methods

Study population
Samples of saliva and serum of 161 children (mean age 6.98 ± 3.09 years) from different rural and urban schools of Venezuela were evaluated. Sixty-six of the children with found infected with *G. duodenalis* by direct microscopy of serially collected stool samples, 22 were found infected with other protozoa (*B. hominis, Entamoeba coli, Entamoeba histolytica, C. mesnilli*) and 73 were found to be non-parasitized.

Saliva samples
Saliva samples were collected by sterile cotton swabs which were maintained in contact with oral mucosae for 10 min. Saliva secretion was recovered by compression in a syringe and kept in tubes with EDTA (Vacutainer) at –20°C.

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.

Antigen preparation
The antigen of *G. duodenalis* was obtained from *in vitro* axenic cultures of *Giardia* trophozoites of the P1 strain (American Type Culture Collection [ATCC], no. 30888) in filter-sterilized TY1-S-33 medium (Keister DB, 1983) containing 10 per cent inactivated bovine serum (Gibco, Grand Island, NY) and bovine vile (Sigma, St. Louis, Missouri). Parasites were cultured for 72 h at 37°C in 5 ml glass-culture tubes. Parasites in late log phase were detached by chilling on ice for 30 min, harvested and centrifuged at 1000 *g* for 20 min at 4°C. The parasites were washed three times with cold phosphate-buffered saline (PBS), pH 7.2, resuspended and lysed in ice-cold PBS containing 0.5 per cent Triton X-100. Protein concentration was measured by the method of Bradford and the antigen was stored at –70°C in aliquots at a concentration of 2 mg/ml.

IgA determinations
Total secretory IgA was measured using a monoclonal antibody against human secretory component diluted at 1:1000 in carbonate-bicarbonate buffer at pH 9.6 and incubated for 2 h at 37°C. The plates were blocked with 10 per cent horse serum-PBS. Saliva samples were diluted 1:500 in 10 per cent horse serum-PBS. After incubation and further washes with PBS-Tween, the plates were incubated with peroxidase-conjugated anti-human-IgA (Sigma) diluted 1:2500 in horse serum-PBS. The washing process was repeated and *o*-phenylenediamine (OPD) plus H_2O_2 were added. Optical density (OD) was read at 490 nm.

The levels of specific secretory and serum IgA were measured by an ELISA developed in our laboratory. *Giardia* antigen (3 µg/well) was diluted in carbonate-bicarbonate buffer at pH 9.6 and coated onto 96-well microtitre plates (Immunolon IV, Dynatech Laboratories Inc, Virginia, USA) and incubated overnight at 4°C. Excess antigen was washed off with PBS-Tween and blocked for 2 h at 37°C with horse serum diluted 1:30 in PBS. The test samples, saliva or sera (100 µl/well diluted 1:5 in 1/30 horse serum-PBS) were plated and incubated for 1 h at 37°C, and peroxidase-conjugated anti-human IgA was used at a dilution of 1:1000.

Statistical analysis
The means and standard deviations were compared by a non-paired *t*-test using the Welch correction. Exact Fisher tests were used to compare proportions. The *p*-values less than 0.05 were considered significant. All tests were performed using GraphPad InStat 3.02 (GraphPad Software, San Diego, CA, USA). Mean OD of serum and secretory specific IgA levels (+2 SD) of the non-parasitized group were considered as cut-off values. The ELISA technique was validated using the direct microscopic stool examination as the gold standard. The sensitivity of the test was calculated as the probability of a given positive value obtained by ELISA, which was also positive by stool examination. Similarly, the specificity of the technique was calculated as the probability of a given negative value obtained by ELISA which was negative by stool examination.

Results

In the present study we quantified the total and specific secretory IgA levels in three groups of children: those parasitized with *G. duodenalis*, those with other protozoa, and non-parasitized children as a control group.

As can be seen in Fig. 1, the total secretory IgA levels were significantly higher in children parasitized with *G. duodenalis* ($p < 0.001$) and with other protozoa ($p < 0.001$) compared with the non-parasitized group. Similar results were obtained when we measured levels of specific IgA in serum (Fig. 2a). The levels of specific slgA were significantly higher in the group of children parasitized with *Giardia*

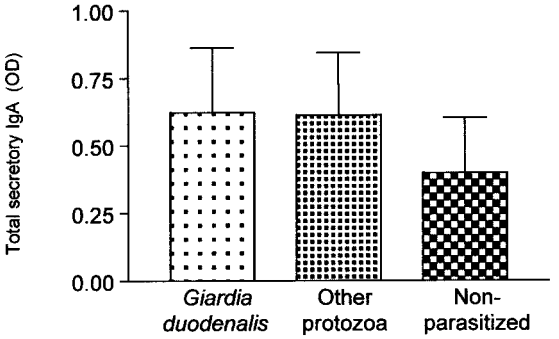

FIG. 1. Total secretory IgA levels in Venezuelan children parasitized and non-parasitized with *G. duodenalis*. $p < 0.001$ for differences between non-parasitized group versus *Giardia duodenalis* and other protozoa group.

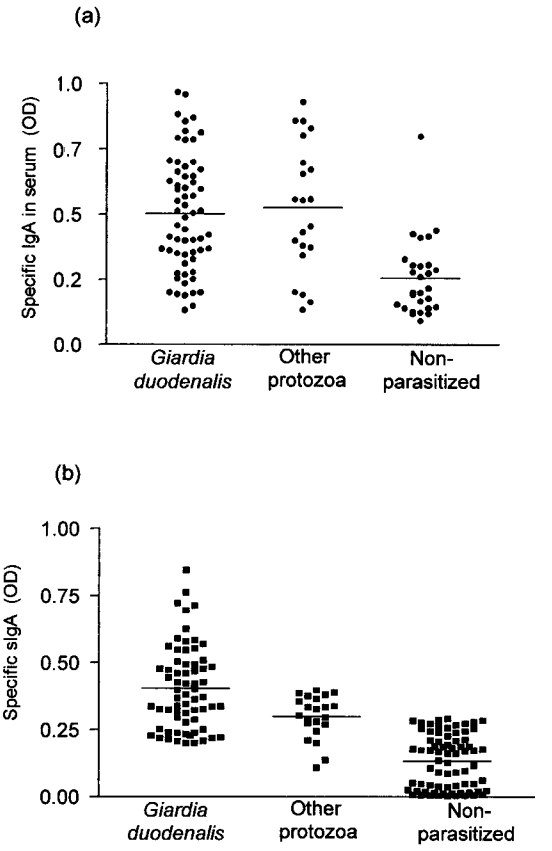

FIG. 2. Specific anti-*G. duodenalis* IgA levels in Venezuelan children parasitized and non-parasitized with *G. duodenalis*. (a) Specific anti-*G. duodenalis* IgA in serum. $p < 0.001$ for differences between non-parasitized group vs. *G. duodenalis* and other protozoa groups. (b) Anti-*G. duodenalis* secretory IgA. $p < 0.001$ for differences between non-parasitized group vs. *G. duodenalis* and other protozoa groups. $p < 0.01$ for differences between *G. duodenalis* vs. other protozoa group. Points represent individual subjects. Crossbars indicate mean IgA levels.

compared with the group parasitized with other protozoa ($p < 0.01$) and the non-parasitized group ($p < 0.001$) (Fig. 2b).

The mean and standard deviation of the specific anti-*Giardia* sIgA values (OD) of the non-parasitized children were determined. The mean (+2 SD) of these values, OD 0.293, was used as the cut-off and samples with higher ODs were considered positive. Based on this criterion of positivity, 49/66 of the children parasitized with *Giardia*, according to direct examination of stool samples, were considered as positive in the ELISA anti-*Giardia* secretory IgA test. In contrast, 4/73 of the non-parasitized children were positive in this ELISA test (Table 1b). The sensitivity and specificity of the test were calculated. They were found to be of 74 and 95 per cent, respectively. The predictive value of a positive test was 92 per cent, and 80 per cent for a negative test.

Likewise, the cut-off for serum specific anti-*Giardia* IgA OD values was found to be 0.476. We found that 35/66 of the parasitized children were positive in the ELISA test (Table 1a), and only one of the non-parasitized children was positive. Similarly, the sensitivity and specificity of the test were 57 and 97 per cent, respectively. The predictive value for a positive sample was 97 per cent and 53 per cent for a negative sample.

Discussion

There is ample experimental evidence demonstrating that the lymphoid tissue associated with the gastrointestinal tract can be activated by parasite-derived antigens such as *Giardia*, which may stimulate specific antibody responses against this parasite, mainly of the IgA isotype.[14,17] Our results show high levels of total secretory IgA in children with protozoal infection, suggesting that different protozoan infections may stimulate the production of this immunoglobulin. Studies performed in day-care centres have reported the utility of sIgA levels for monitoring giardiasis, which has shown to be prevalent among these children.[18] In addition, an inverse relationship between the levels of this antibody and gastrointestinal symptoms associated with this infection has also been reported.[19] In agreement with these findings, our results demonstrated elevated levels of serum and secretory anti-*Giardia* IgA in the group of *Giardia*-parasitized children compared with their non-parasitized counterparts.

TABLE 1
*Specific anti-*G. duodenalis *IgA in serum (a) and anti-*G. duodenalis *secretory IgA (b) levels vs. stool examination in Venezuelan children parasitized and non-parasitized with* G. duodenalis

ELISA	Stool examination		
	Positive	Negative	Total
(a) Serum anti-*G. duodenalis* IgA levels			
Positive	35	1	36
Negative	26	29	55
Total	61	30	91
Sensitivity 35/61 = 57%; specificity 29/30 = 97%			
(b) Anti-*G. duodenalis* sIgA levels			
Positive	49	4	53
Negative	17	69	86
Total	66	73	139
Sensitivity 49/66 = 74%; specificity 69/73 = 95%			

When we compared the *Giardia*-infected group of children with those parasitized with other protozoa, we found significantly higher levels of specific sIgA in the giardiasis group. The levels of serum specific IgA were similar in these groups of children, suggesting that the levels of this antibody may reflect previous exposure to parasitic antigens, rather than current infection.

Confirmation of clinical diagnosis of giardiasis is difficult to assess due to the lack of sensitivity of conventional methods used in the laboratory and the invasive and risky aspects of more sensitive methods.[7,8] A single microscopic examination of stool specimens for evidence of the presence of intestinal parasite only detects 50–70 per cent of the infected patients;[3,20] multiple stool examinations increase this value to over 90 per cent. Repeated microscopic examination of stool specimens can be time-consuming and expensive. Also, its sensitivity can be lower in chronic giardiasis.[20] Several authors have evaluated ELISA techniques for the detection of serum specific anti-*G. duodenalis* antibodies (IgG and IgM) as a diagnostic method.[9,10] However, these methods fail to distinguish current from past infection.[20,21] Other authors have shown the utility of sIgA in the diagnosis of infections caused by other protozoa.[22] In the present study we compared the sensitivity and specificity of ELISA techniques for the detection of serum and sIgA with traditional microscopic examination of stool specimens.

In this work, secretory anti-*Giardia* IgA levels showed better specificity (95 per cent) than sensitivity (74 per cent), probably due to antigenic variability as a consequence of escape mechanisms developed by these parasites.[23] In active infections, a variable proportion of specific antibodies may be attached to the surface of parasites, reducing their concentration in secretions.[14] In younger children, immunological immaturity in the development of IgA responses might contribute to lower sensitivity of the test. It is also possible that the mixture of antigens used does not contain appropriate epitopes, which are able to stimulate high affinity specific IgA antibodies against this parasite.[24] On the other hand, serum specific IgA was found to have significantly less sensitivity than sIgA antibodies. This could indicate that *Giardia* antigens preferentially stimulate the sIgA response.

In summary, our results suggest that secretory anti-*Giardia* IgA levels measured in saliva samples may reflect local intestinal IgA responses elicited by these parasites. The non-invasive nature of collecting samples of saliva represents a clear advantage for studies in children. Although the ELISA technique is not a substitute for the traditional microscopic method, it represents an important alternative tool for the diagnosis of giardiasis when stool examinations are repeatedly negative and the patients exhibit giardiasis-like symptoms. It is of great importance to demonstrate a clear association between detectability of anti-*Giardia* sIgA and active infection. This requires follow-up studies to determine if active infection is clearly associated with anti-*Giardia* sIgA production and if resolution of parasite infection is associated with a reduction of anti-*Giardia* sIgA production.

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