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ANTI-LEISHMANIAL IgE ANTIBODIES: A MARKER OF ACTIVE DISEASE IN VISCERAL LEISHMANIASIS

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Abstract. Visceral leishmaniasis (VL) is characterized by a depression of the T helper cell type 1 immune response. Although mRNA expression for interleukin-4 (IL-4) is observed, evidence of the role of this cytokine in the pathogenesis of VL has been lacking. Since IL-4 is involved in IgE synthesis, we measured the total IgE and *Leishmania* antigen-specific IgE antibody levels in sera from patients with VL. Specific IgE antibodies detected by an ELISA technique after absorbing the sera with purified sheep IgG anti-human IgG were found in all 23 patients with VL and were not detected in subjects with subclinical *Leishmania chagasi* infection (n = 10), Chagas' disease (n = 10), atopic patients (n = 10), and healthy controls (n = 10). Levels of *Leishmania*-specific IgE (optical density values) before and after treatment were 0.100 ± 0.03 (mean ± SD) and 0.028 ± 0.002, respectively ($P < 0.05$). These results indicate that a specific IgE response is useful in the diagnosis of active disease and to evaluate response to treatment.

Immunity to leishmanial infection is cell-mediated and results in the killing of intracellular organisms by macrophage activation and cytotoxic responses.[1–3] Failure to control intramacrophage growth of *Leishmania chagasi,* the causal agent of American visceral leishmaniasis (VL), leads to a severe disease associated with hepatosplenomegaly, pancytopenia, hemorrhagic complications, and increased susceptibility to microbial infections.[4] High titers of antibody are found in the course of *L. chagasi* infection and antibody detection is an important diagnostic tool in identifying cases of VL[5–7] and individuals with subclinical *L. chagasi* infection.[8–10] Only a few studies have investigated the immunoglobulin isotype responses elicited during the course of VL. In such cases, production of IgM, IgA, and the IgG subclasses have been documented during disease and an IgG1 isotype response appears to predominate.[11, 12]

The T cell response is different in individuals with subclinical self-healing *L. chagasi* infections and patients with VL. While lymphocytes from subjects who are able to control their *L. chagasi* infections produce interferon-γ (IFN-γ) upon stimulation with leishmanial antigen *in vitro,*[13] the immunologic response in patients with VL is characterized by an absence of interleukin-2 (IL-2) and IFN-γ production,[14, 15] and a high level of IL-10 mRNA expression and IL-10 production.[16–19] Although dissemination of leishmanial infection in mice is dependent on IL-4,[20] evidence for IL-4 production in human VL is minimal or absent.[19, 21] Since IL-4 is the primary cytokine that induces T helper cell type 2 (Th2) activation and B cell differentiation leading to IgE synthesis,[22, 23] we measured levels of total IgE and specific anti-*Leishmania* IgE antibodies in individuals with different clinical forms of VL and determined whether these levels were associated with disease severity, levels of IgG antibodies, and response to therapy.

PATIENTS, MATERIALS, AND METHODS

Study patients. Participants of this study included 23 patients with VL, 10 subjects with subclinical *L. chagasi* infection, 10 atopic patients with a history of asthma and/or allergic rhinitis, 10 patients with Chagas' disease, 10 subjects living in an endemic area of *L. chagasi* transmission, but not exposed to *Leishmania,* and 10 healthy subjects living outside the endemic areas. Patients with VL were recruited from two hospitals (Hospital Santo Antonio and Hospital Universitario Professor Edgard Santos) located in Salvador, Bahia, Brazil, and all of them had documented *Leishmania* amastigotes in Giemsa-stained bone marrow or splenic aspirates. The subjects with subclinical infections, the individuals living in the endemic area but not exposed to *L. chagasi,* and nine of the 23 patients with VL were from the endemic area of Monte Gordo, a village in the state of Bahia in northeastern Brazil. An outbreak of VL occurred in this village in 1989 and epidemiologic, clinical, and immunologic studies have been conducted in this area.[24] Sera collection for the determination of antibodies to *L. chagasi* is performed annually and seroconverting subjects are considered to be infected by *L. chagasi.* Follow-up clinical evaluation of the infected subjects is performed to determine if they develop any symptoms or signs of VL. Patients with subclinical infection were defined as those who had documented seroconversion between 1993 and 1994 and remained asymptomatic until 1996.

This investigation was part of studies approved by the Ethical Committee of the Hospital Universitário Professor Edgar Santos. Informed consent, following the guidelines of the Brazilian Ministry of Health for research with human subjects, was obtained from all patients.

Preparation of leishmanial antigen and detection of anti-leishmanial IgG and IgE antibodies levels. Soluble antigen was prepared from a cloned strain of *Leishmania* (MHOM-BR 86-BA) that was isolated from a patient with VL and has been identified by monoclonal antibodies, isoenzymes, and kinetoplast DNA analyses to be *L. chagasi.* Leishmanial antigen was prepared from 10^9 stationary phase promastigotes that were washed three times washed in phosphate-buffered saline (PBS) and lysed with 6 mM CHAPS (3-[(3-cholamidopropy) dimethylammonio]-1-propane sulfonate) in Tris-HCI buffer containing 150 mM NaCl. This material was centrifuged (6,500 × *g*) for 5 min, after which the supernatant was collected and stored at −20°C until use.

TABLE 1
Clinical profile and IgE antibodies in visceral leishmaniasis*

Patient no./name	Age (years)	Illness duration (days)	Spleen size (cm)	Ht (%)	WBC/ mm²	Eosinophils/ mm²	Parasites in stool	Total IgE (IU/ml)	*Leishmania chagasi* IgE specific†
K.10/AJM	1	30	1	21	3,900	117		1,400	0.120
K.11/ASO	2	30	10	14	1,500	45	Gl	877	0.088
K.16/MNM	2	180	13	25	4,000	0	Tt	596	0.102
K.05/FOS	2	8	7	21	3,100	31	–	1,329	0.122
K.15/RBB	3	60	4	30	6,700	0	Ad	246	0.120
K.03/MFB	3.5	60	8	21	2,300	0	–	170	0.097
K.02/JBB	5	45	10	15	2,100	0	Gl	>4,000	0.090
K.01/AOM	6	60	13	25	4,200	84	Ec	>4,000	0.093
K.14/GOS	6	90	13	28	3,400	0	–	1,596	0.089
K.04/VSS	7	14	9	31	3,500	35	–	1,791	0.111
K.09/LJS	7	240	13	12	3,000	60	Ad	>4,000	0.106
K.07/FMS	8	30	6	26	4,100	ND	Ss	1,359	0.113
K.06/SJS	9	180	6	30	3,100	589	–	2,063	0.054
K.12/CSS	12	90	13	31	3,500	0	-	265	0.141
K.13/GVJ	13	21	7	28	2,800	28	Ad	162	0.110
K.20/GJ	17	120	20	33	3,000	0	–	54	0.097
K.21/JAC	22	40	NP	22	2,300	46	–	54	0.176
K.19/JCJ	24	365	16	28	2,800	56	–	1,396	0.103
K.08/IBS	30	365	23	24	1,500	ND	Tt, Ec, Eh, Al	180	0.114
K.18/TNS	37	150	12	21	1,400	42	Tt	1,967	0.118
K.23/RMO	38	15	5	33	2,900	29	–	967	0.152
K.17/LES	47	330	15	19	1,900	0	Tt, Al, Sm	2,279	0.085
K.11/MBJ	49	90	5	13	1,300	13	Ad	904	0.070

* Ht = hematocrit; WBC = white blood cells; Gl = *Giardia lamblia*; Tt = *Trichuris trichiura*; – = negative; Ad = *Ancylostoma duodenale*; Ec = *Entamoeba coli*; ND = not done; Ss = *Strongyloides stercoralis*; NP = not palpable; Eh = *Entamoeba histolytica*; Al = *Ascaris lumbricoides*; Sm = *Schistosoma mansoni*.
† The specific IgE cut-off value was 0.027.

The protein content of the lysate was determined by the Bradford method.[25]

To detect IgG antibodies against *Leishmania* antigens, an indirect ELISA was performed using polystyrene microtiter plates previously coated with 500 ng of parasite protein per well. After incubation with 1% bovine serum albumin (BSA) in PBS and washes in PBS, 0.05% Tween, the microliter wells were incubated with 100 μl of sera diluted 1:100 for 30 min at 25°C. The wells were washed three times with PBS-0.05% Tween and then incubated with 100 μl of goat anti-human IgG peroxidase conjugate as above. The wells were washed again and the color was developed for 15 min with chromogenic solution (3, 3′, 5, 5′-tetramethylbenzidine-hydrogen peroxide). After stopping the reaction with 2 N HCI, absorbances were measured at 450–600 nm using a Diamedix (Miami, FL) microassay BP-12 ELISA reader.

The ELISA to detect anti-*L. chagasi* IgE was performed using sera preincubated with purified sheep IgG anti-human IgG (RF absorbent; Behring Diagnostics, Marburg, Germany) to eliminate IgG antibody competition. Fifty microliters of the sera were mixed with 100 μl of RF absorbent for 1 hr at 25°C and then centrifuged at 6,500 × *g* for 5 min to remove the immune complexes. For the assay, 100 μl of supernatants diluted 1:2 in 50 mM Tris-HCI buffer (pH 7.5) containing BSA were added to wells coated with *L. chagasi* antigens. After incubation for 18 hr at 40°C, the wells were washed with PBS and incubated with 100 μl of goat anti-human IgE peroxidase conjugate for 30 min at room temperature. The solid phase was washed again and the reactions were developed as described above. All sera were processed and analyzed for detection of IgE on the same day.

Western immunoblotting. Identification of specific leishmanial antigens recognized by IgE antibodies from patient sera was performed using Western immunoblotting. One hundred fifty microliters of protein lysate were electrophoresed on a sodium dodecyl sulfate–12.5% polyacrylamide minigel using the method of Laemmli.[26] After electrophoresis, the fractionated polypeptides were transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon; Millipore Corp., Bedford, MA) at 300 mA for 1 hr at 4°C. After transfer, free sites were blocked with PBS containing 0.5% Tween 20 for 1 hr at room temperature. The primary reaction was performed by incubating strips of PVDF membrane for 18 hr at 4°C with 500 μl of sera that was previously depleted of human IgG by diluting the sera 1:5 in PBS with 25% (v/v) protein G-Sepharose. The strips were washed with 50 mM Tris-HCI buffer (pH 7.5) containing 150 mM NaCl and 0.05% Tween 20 and incubated for secondary immune reaction with diluted goat anti-human IgE-alkaline phosphatase conjugate (Sigma, St. Louis, MO) for 1 hr at 37°C. The blots were then washed with the same buffer and developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate; Sigma).

Total serum IgE. The IgE concentration in sera from controls and patients with VL was determined by an antigen-capture ELISA (Medix Biotech Inc., San Carlo, CA) using an anti-human IgE monoclonal antibody and a goat anti-human IgE peroxidase conjugate. The results are expressed in International Units (IU).

RESULTS

The clinical profile of the 23 patients with VL is shown in Table 1. The age of the patients ranged from one to 49 years and the duration of illness ranged from eight days to one year. Anemia was observed in all patients and leuko-

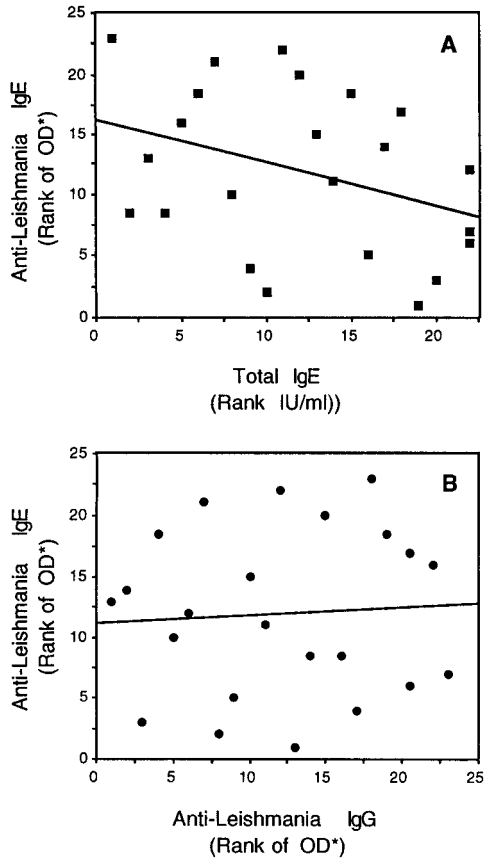

FIGURE 1. **A,** absence of a correlation between anti-*Leishmania* IgE and total IgE in 23 patients with visceral leishmaniasis. $P > 0.05$, by Kendall rank correlation test. **B,** absence of a correlation between anti-*Leishmania* IgE and anti-*Leishmania* IgG in 23 patients with visceral leishmaniasis. $P > 0.05$, by Kendall rank correlation test. OD* = optical density.

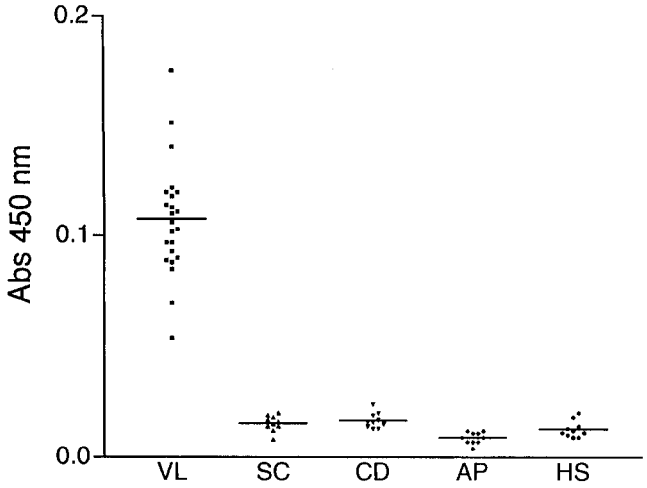

FIGURE 2. Specific *Leishmania chagasi* IgE antibodies detected by ELISA in sera from patients with visceral leishmaniasis (VL), subjects with subclinical *L. chagasi* infection (SC), patients with Chagas' disease (CD), atopic patients (AP), and healthy subjects living in endemic area not exposed to *L. chagasi* infection (HS). Abs = absorbance.

penia (white blood cell counts $< 4{,}000$ cells/mm^3) was present in 19 (88%) of the patients. Eosinophil counts were normal or absent in the blood smears of all but one patient. All patients but one had a palpable spleen below the left costal margin on examination. Intestinal helminthic infections were documented in 10 (43.4%) patients.

The mean ± SD total IgE levels in patients with VL (1,376 ± 1,245 IU) were higher than ($P < 0.05$) that observed in healthy subjects living outside the endemic area (92 ± 26), but were not statistically different from those observed in patients with atopic disease (957 ± 627 IU/ml), in subjects with subclinical *L. chagasi* infection (627 ± 543 IU/ml), and in individuals living in the endemic area but not exposed to *L. chagasi* infection (721 ± 625 IU/ml).

Specific anti-leishmanial IgE was not detected in the ELISA when whole serum was used due to the presence of blocking IgG antibodies. However, when serum was preincubated with RF absorbent, a reagent that blocks IgG binding sites and depletes IgG, we were able to detect binding of specific anti-leishmanial IgE in sera from all patients with VL. The mean ± SD total IgG levels measured by radial immunodifusion in sera from three patients with VL was 2,782 ± 612 mg/dL. After mixing with RF absorbent, only IgG levels decreased to 1,092 ± 230 mg/dL, suggesting that the main effect of RF absorbent was to block IgG binding sites. The range of optical densities (ODs) in sera from patients with VL was 0.070–0.176. There was no statistically significant association between total IgE or specific IgE and duration of illness, spleen size, anemia, and numbers of neutrophils. The sera of all 23 patients with VL had high levels of specific IgG to *L. chagasi* with ODs ranging from 0.554 to 1.266. No correlation was identified between *L. chagasi* IgG and IgE levels ($P > 0.05$) or total IgE and specific IgE levels (Figure 1).

The OD for specific IgE antibodies to leishmanial antigens in the different groups studied is shown in Figure 2. The mean ± SD OD in patients with VL (0.108 ± 0.026) was higher than that observed in the subjects with subclinical *L. chagasi* infection (0.015 ± 0.003; $P < 0.05$). In the control group, specific IgE antibody ODs were as follows: Chagas' disease (0.016 ± 0.003), atopic patients (0.021 ± 0.003), healthy subjects living in the endemic area (0.012 ± 0.004), and healthy subjects living outside the endemic area (0.013 ± 0.001). The sensitivity and specificity of the test for IgE antibody to *L. chagasi* for the diagnosis of VL were 100%.

Western immunoblotting was performed with sera of three patients with VL. The IgE antibodies predominantly recognized antigens of with M_r values of 96, 67, and 46 kD. Specific IgE antibodies were evaluated in seven patients before and after (1–3 months) therapy for VL (Figure 3). The mean OD ± SD in these patients was 0.100 ± 0.033 before therapy and 0.028 ± 0.002 ($P < 0.05$) after cure.

DISCUSSION

Production of IgE antibodies is frequently associated with helminthic disease and allergy. These antibodies have been reported to play a role in the host defense mechanism in helminthic diseases and in tissue damage secondary to mast cell degranulation in allergy.[27–30] Recently, induction of an IgE antibody response has been documented during infec-

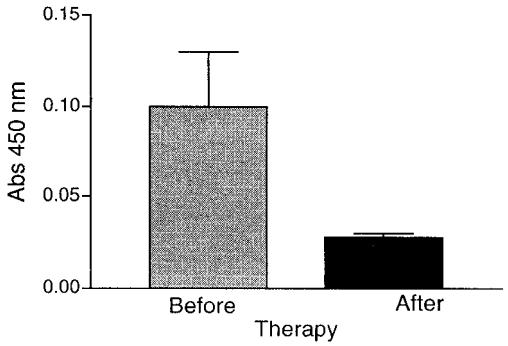

FIGURE 3. Anti-leishmanial IgE antibodies before and after treatment of patients with visceral leishmaniasis. Values on the y-axis are optical density units. Bars show the mean ± SD.

tions with intracellular pathogens, and measurement of IgE antibodies has been used as a diagnostic or prognostic marker in protozoa or viral diseases.[31–33] For example, high levels of total IgE are associated with low CD_4+ counts and disease progression patients infected with human immunodeficiency virus,[33] and with cerebral complications in patients with malaria.[32]

Visceral leishmaniasis is associated with high levels of circulating immunoglobulin, polyclonal B cell activation,[34] and marked expression of mRNA for IL-4.[17, 19] One potential consequence of these components of the immunologic response during the evolution of the disease is the production of IgE. In this study, elevation of total IgE levels was observed in patients with VL, but was also documented in subjects with subclinical *L. chagasi* infection and in individuals living in the endemic area without exposure to *L. chagasi* infection. Helminthic infections are prevalent in northeastern Brazil and may explain the high levels of IgE in individuals without infection or disease who reside in the endemic areas.

In contrast to the results for total IgE levels, specific IgE antibodies were only observed in patients with VL. This finding indicates that production of specific anti-leishmanial IgE antibodies are not merely a consequence of a polyclonal B cell activation, but rather an antigen-driven response. The lack of a positive correlation between total and specific IgE and specific *L. chagasi* IgG and IgE antibodies further supports this argument. The underlying mechanism may involve an immunoglobulin class switch to IgE after specific recognition of leishmanial antigens and induction by IL-4 during the evolution from infection to disease.

Although IL-4 production is associated with increased parasite burden and dissemination of disease in the murine model of leishmaniasis,[35] IL-10 has been the main cytokine associated with the down-regulation of the Th1 response and pathogenesis of the disease in human VL.[16–19, 36] Documentation of high levels of specific IgE indicates that this immunoglobulin class is a marker of Th2 activation in VL.

Detection of IgG antibodies to *L. chagasi* have been applied to the diagnosis of VL. However, these antibodies have been observed to cross-react with antigens of other infectious agents, particularly *Trypanosoma cruzi*,[37] and are found in individuals with subclinical *L. chagasi* infection,[8–10] and persist after successful therapy for VL.[5, 18] In the present study, the sensitivity and specificity for the anti-leishmanial IgE detection assay in diagnosing VL were 100%. More importantly, specific IgE antibodies are a marker for active disease. In contrast to IgG antibodies, they are not present in subclinical infections and their levels disappear rapidly after treatment of VL.

T cell abnormalities, as detected by tests measuring mRNA levels for IL-4 and IL-10, IFN-γ production, and lymphocyte proliferation, are reversed after therapy for VL.[14, 19, 38] The cost and feasibility of performing these tests limit their application as a marker of therapeutic response in clinical practice. Our findings indicate that anti-leishmanial IgE antibodies are a sensitive and specific marker for active disease, and that the ELISA is a lower cost alternative that can be easily performed and yield rapid results. Further studies are needed to confirm the validity of this test in other endemic sites of VL.

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