Detection of Specific Antibody Isotypes and Subtypes Before and After Treatment of American Visceral Leishmaniasis

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Sera from patients with American visceral leishmaniasis (AVL) were studied before and after treatment based on their antibody isotypes and subtypes. The study was comprised of 33 Brazilian patients with well-defined diagnosis of AVL and 39 clinically healthy individuals. Antileishmanial antibody isotypes and subtypes were observed in almost all patients, except IgA that was detected in about 63% of them. The sensitivity and specificity of the immunofluorescence assay in the detection of antibody isotypes (IgG and IgM) and subtypes (IgG1, IgG2, IgG3, and IgG4) were high with no statistical difference, ranging from 0.937 to 1.000 and from 0.954 to 1.000, re-

spectively. All IgG antibodies and its subtypes had their levels reduced after treatment. However, the IgG4 had an early decay and its conversion to negative was significantly high in children. Moreover, the profile of IgG4 before treatment corresponded to a unimodal curve that shifted to a patent bimodal curve after treatment, indicative of therapeutic success. Thus, the IgG4 shows to be a suitable immunological marker for the assessment of chemotherapy in AVL patients or communities. Our findings suggest that IgG4 correlates with IL-4 that also decreases after therapy. J. Clin. Lab. Anal. 14:5–12, 2000.

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INTRODUCTION

American visceral leishmaniasis (AVL) is a severe and often fatal parasitic disease caused by a systemic protozoan, *Leishmania (L.) chagasi* and is mostly prevalent in the north-eastern region of Brazil (1). The main symptoms of the disease are fever, weight loss, anemia, and hepatosplenomegaly, and death is frequent in untreated cases (2).

The active visceral leishmaniasis was shown to be associated with activation of Th2 cells (3,4). After treatment the cell-mediated immunity is restored and IFN- γ is capable to activate macrophages to kill parasites (5,6). Despite the usual hypergammaglobulinaemia in patients with AVL, antibodies do not seem to play a central role in disease control (7).

The current diagnosis of AVL is based mostly on clinical data and on the parasite demonstration in bone marrow and/or splenic aspirates by direct smear or culture methods. When the parasite is not found, antibody-based serologic methods such as immunofluorescence, direct agglutination, and immunoenzymatic assays have proved to be useful for the diagnosis of leishmaniasis as well as for epidemiological population surveys (8–11). All these methods present relatively high sensitivity and specificity.

To date, the criterion adopted for establishing the efficacy of AVL treatment depends on the clinical improvement of patients and negative result in parasitological methods. Changes in serological patterns have also been suggested to be helpful for the evaluation of patients submitted to chemotherapy (12–17).

Antibody isotypes and subtypes have been found in patients with classical Old World kala-azar or American visceral leishmaniasis (AVL) caused by *Leishmania donovani* (18–20) and by *L. chagasi* (21,22). Nevertheless, antibodies may differ qualitatively and/or quantitatively in patients from different geographical localities related to ethnic/genetic factors and *Leishmania* strains (19,23).

Data on humoral immune response with respect to anti-

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body isotypes and subtypes in *L. chagasi* infection are very scarce. Furthermore, this infection shows clinical, immunological and epidemiological differences from the *L. donovani* infection (2).

Thus in the present work, antibody isotypes (IgG, IgM, and IgA) and IgG subtypes (G1, G2, G3, and G4) were investigated in sera from patients with defined diagnosis of AVL both before and after treatment.

MATERIALS AND METHODS

Collection of Sera

A total of 92 serum samples were collected from 33 patients with clinical and parasitological diagnosis of AVL. These patients were living in endemic areas (States of Espírito Santo and Bahia, Brazil) and their clinical data showed that symptoms of the disease had started from 20 days to 3 months before the time of serum collection. In the follow-up study performed in 22 AVL patients, sera were collected before, during, and immediately after treatment (1 to 20 days) and 1 to 22 months thereafter.

A standard regimen of antimony was given to all patients comprising 19 adults and 14 children. Serum samples were also taken from 39 clinically healthy individuals (CHI) living in the above endemic areas and nonendemic areas for AVL (São Paulo, Brazil) as controls. They were known to be serologically negative for syphilis, hepatitis, Chagas' disease, and HIV infection. Serum samples were stored at -20°C until used.

Antigen Preparation

L. chagasi (MHOM/BR/72/LD46) promastigotes were cultured in RPMI medium (Sigma, St. Louis, MO) supplemented with fetal calf serum containing antibiotics, at 25°C . Parasites at stationary growth phase were harvested and washed 3 times in 0.01M phosphate buffered saline solution, pH 7.4 (PBS). The pellet was resuspended in 2% formalin-PBS and incubated overnight at room temperature. Then parasites were washed as before and resuspended to a concentration of 1.5×10^6 parasites/ml. A volume of 10 μl of the parasite suspension was placed on each delimited area of microscope slides and dried at 37°C . The slides were stored at -20°C until used.

Detection of Antibody Isotypes and IgG Subtypes

An indirect immunofluorescence assay (IFA) was used for the detection of antibody isotypes and IgG subtypes. Briefly, the above promastigote antigen was incubated with serial double dilutions of serum samples from 1:2 to 1:16,300 for 30 minutes at 37°C . After washing in PBS, the slides were incubated with fluorescent conjugates to human IgG and IgM (Biolab S.A, Brazil) and IgA (Behring, Germany), at dilutions of 1:100, 1:100, and 1:20, respectively. Subtypes of IgG were detected incubating the slides with mice monoclonal antibodies to human IgG1 (1:3,000), IgG2 (1:1,000), IgG3 (1:1,000), and IgG4 (1:1,000)

(Unipath Ltd., England), for 45 minutes at 37°C . Then, these subtypes were revealed with a fluorescent conjugate to mouse IgG (1:40) (Cappel-Organon Teknika Corp, USA) by incubating for 30 minutes at 37°C .

Evaluation of Antibody Isotypes and IgG Subtypes as Immunological Markers for the Serodiagnosis and Treatment Monitoring of AVL Patients

The possibility of one of the antibody isotypes or IgG subtypes to be an efficient immunological marker for the diagnosis of AVL was investigated as well as its potential for use in therapeutic monitoring. Thus, the diagnostic performances of seven assays were evaluated in terms of sensitivity, specificity, and positive and negative predictive values in the study of patients before treatment. The antibody decay after treatment was studied in terms of geometric mean of titers (GMT) and in units as defined in the Statistical Analysis. In population surveys, easy methods to evaluate the treatment efficacy are required. We adopted here a model that was proposed for an unrelated parasitic infection (24), by plotting antibody titers against their frequencies. In the successful treatment, three different types of curves were consecutively observed: the unimodal curve (1), which is typical for the period before treatment, changes to a bimodal curve (2) after treatment in account of the antibody titer decays and seroconversions to negative, and becomes unimodal (3) again similar to that presented by an uninfected group of individuals.

Statistical Analysis

The antibody titers were transformed in $\log_2$ (titer = 2^x) to calculate geometric mean titer (GMT). The 95% confidence intervals were calculated (Table 1) and to compare unpaired titers before and after treatment, the Mann-Whitney test was used (Table 2). To analyze seroconversion to negative and titer decay, Fisher's exact test was employed (Table 3). SigmaStat version 1.01 was used for statistical calculations.

The antibody decay was also represented in terms of units (U) as follows: $U = \log_2$ antibody titer after treatment/ $\log_2$ antibody titer before treatment. According to this equation, the antibody titer before treatment will correspond to 1 U. In other words, the qualitative and quantitative antibody levels prior to treatment were considered to be 100%.

The curves corresponding to the frequency of antibody titer distribution before and after treatment were obtained by nonlinear regression of a polynomial equation of the fourth order using the GraphPad Prism version 2.01 (Fig. 2).

RESULTS

Antibody Isotypes and IgG Subtypes in Untreated AVL Patients and Control Groups

Results obtained in the group of patients with AVL and in the group of CHI are presented in Figure 1. The IgG and IgG4

TABLE 1. Diagnostic features of immunofluorescence assay in the detection of antibody isotypes and subtypes for the diagnosis of American visceral leishmaniasis

Isotype/ subtype	Sensitivity (%)	Specificity (%)	Positive PV ^a (%)	Nevative PV (%)	Level in GMT (sd) ^b	
					(AVL) ^c	(CHI) ^d
IgG	100.0	100.0	100.0	100.0	10.8 (1.5)	2.6 (0.5)
IgG1	96.7	100.0	96.7	96.1	8.8 (2.4)	2.6 (0.5)
IgG2	100.0	95.4	96.8	95.4	7.7 (2.5)	1.5 (1.2)
IgG3	100.0	95.4	96.7	95.4	8.6 (1.9)	1.7 (1.2)
IgG4	100.0	100.0	100.0	100.0	8.3 (2.2)	1.6 (1.1)
IgM	93.7	97.2	90.9	92.3	6.1 (.15)	2.5 (0.9)
IgA	63.3	100.0	63.3	77.5	2.7 (2.6)	1.3 (0.5)

^aPredictive value.^bGeometric mean titers and standard deviation.^cAmerican visceral leishmaniasis.^dClinically healthy individuals.

antibodies better discriminated infected from uninfected groups of patients than did other subtypes, which showed some false positive and/or false negative results. In general, IgA and IgM antibodies gave lower titers and more false negative results in comparison with IgG and its subtypes.

The antibody titers were expressed in terms of GMT (Table 1), and the IgG antibody showed the highest (10.8) value in the group of patients with AVL, followed by IgG1, IgG3, IgG4, IgG2, IgM, and IgA. The control group in turn gave a low GMT below cutoff titer, not exceeding the value of 2.6. The GMT of IgG4 antibodies from AVL patients was comparatively 5.2 times higher than that of the control group, and was higher than that of antibodies IgG2 (5.1), IgG3 (5.0), IgG (4.1), IgG1 (3.4), IgM (2.4), and IgA (2.1).

Maximum (100%) sensitivity, specificity, and predictive values of positive and negative (Table 1) were found for IFA in the detection of IgG and IgG4 antibodies. However, these values did not statistically differ from the assay in the detection of IgM and IgG subtypes, when the values with 95% confidence intervals were compared. On the other hand, the assay for IgA antibody detection gave low sensitivity, in spite of the high specificity.

Immunoglobulin G and its Subtypes in AVL Patients Submitted to Treatment

The levels of IgG and its subtypes decayed after the patients' treatment and these levels are expressed in terms of conventional GMT and in units (Table 2). The values of antibody levels presented in units give a better idea of the kinetics of antibody decays. So, before treatment, IgG and its subtypes had levels of 1 U and during the treatment, all levels of antibody types slightly increased (>1 U). However, IgG4 was the only antibody subtype that presented a slightly diminished level immediately after treatment. The period after treatment varied from 1 to 22 months and the levels of all types of antibodies significantly ($P < 0.01$) decreased if compared with their levels before treatment. Particularly, IgG4 antibodies decreased more pronouncedly after treatment than did other IgG subtypes.

In general, curves of the titer frequency obtained for IgG and its subtypes in the period before treatment differed from curves after treatment. Nevertheless, the IgG4 curve presented a clear and significant change after treatment. A unimodal curve was observed before treatment when the frequency of

TABLE 2. Levels of specific IgG and its subtypes before, during, immediately after, and after chemotherapy of patients with American visceral leishmaniasis

Isotype/ subtype	Level							
	Before treatment		During treatment		Immediately after treatment ^a		After treatment ^b	
	(GMT) ^c	(U) ^d	(GMT)	(U)	(GMT)	(U)	(GMT)	(U)
IgG	10.8	1.00	11.1	1.03	11.0	1.02	7.4	0.69
IgG1	8.8	1.00	9.6	1.09	9.5	1.08	6.4	0.73
IgG2	7.7	1.00	8.4	1.09	7.9	1.03	4.1	0.53
IgG3	8.6	1.00	9.5	1.10	9.2	1.07	4.4	0.51
IgG4	8.3	1.00	8.4	1.01	8.0	0.96	3.7	0.45

^a1 to 20 days post-treatment.^b1 to 22 months post-treatment.^cGeometric mean titers.^dUnit.

TABLE 3. Antileishmanial IgG4 antibodies found in paired samples from 19 patients with American visceral leishmaniasis after submitted to treatment

Patient (n)	GMT ^a (U) ^b		IgG4 Levels after treatment ^c		
	Before treatment	After treatment	Conversion to negative	Significant decay	No decay
Adults (8)	8.7 (1.00)	4.4 (0.57)	2 (25%)	4 (50%)	2 (25%)
Children (11)	7.7 (1.00)	2.1 (0.27)	7 (64%)	1 (9%)	3 (27%)
Total (19)	8.1 (1.00)	2.8 (0.35)	9 (47.4%)	5 (26.3%)	5 (26.3%)

^aGeometric mean titers.^bUnits.^c1 to 22 months.

antibody titer was studied by a polynomial equation of the fourth order. However, after treatment this curve shifted to a bimodal curve because about 47% of the patients had IgG4 conversion to negative and about 26% of them had their antibody levels significantly decayed (≥ 2 titers). In Figure 2, these two types of curves are presented along with the antibody frequencies obtained in this study. The antibody conversion to negative was about 32% for IgG and IgG1, and 22% for IgG2 and IgG3—all lower levels than for IgG4.

Immunoglobulin G and its subtypes had no significant decay in about 21–32% of patients.

Immunoglobulin G4 in Paired Samples From Adults and Children Before and After Treatment

The features of IgG4 antibodies were studied in children and adults, taking paired samples before and after treatment

from 19 AVL patients—8 adults and 11 children. The decay of IgG4 antibody levels after treatment was evident in both groups. However, the seroconversion to negative was significant ($P < 0.01$) only in children (Table 3).

Correlation of Clinical and Parasitological Data of AVL Patients Submitted to Treatment With IgG4 Antibodies

All treated AVL patients ($n = 33$) showed a clinical improvement and had negative results by parasitological assay; these data are conventionally adopted as criteria for cure. Immunoglobulin G4 antibodies showed better correlation with these criteria than did other types of antibodies. In brief, IgG4 had an earlier and greater antibody decay after treatment (Table 2). Moreover, about 73% from the 19 followed-up patients had IgG4 patterns that had changed considerably after treat-

Fig. 1. Scatter diagrams of specific antibody isotypes in patients with American visceral leishmaniasis (AVL) and in clinically healthy individuals (CHI). Dashed lines (---) define the cutoff titer for each.

Fig. 2. Frequency and distribution of IgG4 antibody titers in American visceral leishmaniasis. Antibody titer frequencies obtained before treatment ($\square$) and after treatment ($\circ$); curves corresponding to before (---) and after (-----) treatment according to a polynomial equation of the fourth order; theoretically expected curve (—) for cured patients, similar to the clinically healthy individuals.

ment represented by a significant conversion to negative and decay in its level (Table 3).

DISCUSSION

In the diagnosis of AVL, serological assays are more sensitive and less invasive and risky than the parasitological methods, although the demonstration of parasite in clinical specimens provides true diagnosis. In the present work, the high humoral immune response in AVL patients demonstrates the advantage of using serum samples to diagnose clinical cases, perform epidemiological surveys, and monitor patient and community therapy.

Prior to the specific chemotherapy, IgG and its subtypes were detected in almost all AVL patients. However, IgM and IgA, found in low titers, were not present in 6. % and 36.7% of patients, respectively (Table 1). The IgM and IgA GMT were observed to inversely correlate with the clinical findings of the patients before treatment. Patients with a short period (up to 1 month) of symptomatology showed 7.0 GMT for IgM and 4.3 GMT for IgA antibodies. Nevertheless, the GMT of the same antibodies decreased to 6.0 and to 3.7 from 1 to 3 months of symptoms, respectively (data not shown). These findings show that IgM and IgA antibodies are related to the early phase of the infection, and it is expected that low titers or negative results will be found thereafter.

The IgM antibodies were detected in 93.7% of AVL patients, similar to the Indian VL patients (25), but this

isotype was absent in Sudanese VL patients (18) who usually informed a long history of infection before the time of their serum collection.

In unrelated parasitic, bacterial, or viral infections, IgM is usually considered to be an immunological marker for the acute phase of infection, but in some parasitic infections such as schistosomiasis, this isotype can also be detected in the chronic phase of the infection with serodiagnostic value (26).

The IgA antibodies were found in 63% of AVL patients, in contrast to the Sudanese patients whose IgA isotype was absent (18).

The prognostic value of total or specific IgA in active AVL patients has to be further investigated because it seems to correlate with severe clinical forms in some diseases such as mucocutaneous leishmaniasis (27,28), the digestive form of chronic Chagas' disease (29), and HIV infection in drug users (30).

In all AVL patients, high levels of IgG antibodies were detected confirming previous data obtained by other investigators using different types of immunoassays in the diagnosis of VL (11,31,32). Subtypes of IgG were also found in all AVL patients and our findings partially agree with those observed in the VL patients; in the latter patients only IgG1 and IgG3 were consistently detected (18–20). On the other hand, the presence of IgG2 in VL patients has been controversial since it was found by some authors (19,20), but not detected by others (18).

Interferon-gamma can be detected in active VL patients (33,34), and this cytokine promotes human IgG2 production (35). In canine VL, specific IgG2 was observed in symptomatic dogs but it was maintained at high levels after successful therapy, in contrast to IgG1 which had decreased (36).

The IgG4 antibodies were demonstrated in all patients with AVL, in contrast to the VL patients, who had very little or none (18,20) or even up to 67% (19).

Active VL has been associated with hypergammaglobulinaemia and elevated Th2-cytokines (3,4,33), inducing high B-cell response. In addition, IL-4 preferentially enhances IgG4 and IgE synthesis whereas IL-10 induces IgG1/IgG3 synthesis (37–39). In our study these IgG subtypes were significantly elevated and in terms of GMT, they were ranked in the following order: IgG1 > IgG3 > IgG4.

Immunoglobulin E antibodies were not included in this study, but they had also been detected in AVL patients (22).

Brazilian AVL patients seem to show a different pattern of immune response in relation to Venezuelan AVL patients as, in the latter, IgG4 is mostly found in low levels, though in some patients these levels are seen to be significantly high. Moreover, IgG2 and IgG3 are absent in patients from Venezuela (21). Antigens or epitopes of *L. chagasi* from different strains may be responsible for inducing a different pattern of immune response.

There are factors such as parasite isolates, strains, and genetic components of the population, as well as the duration and severity of the disease which influence the serology, giv-

ing discrepant data. However, the immunoassay systems also seem to be additional factors to be taken into account. In the ELISA system, for instance, formalin-treated promastigote antigens (18,21) and crude parasite extract antigens (19–21) are used, whereas in the IFA, formalin-treated promastigote antigens are employed. So, antileishmanial IgG1 and IgG3 react with protein antigens found in both types of antigens. Nevertheless, IgG2 antibodies react mostly with polysaccharide antigen (40) present in the membrane of intact parasite. On the other hand, this antibody subtype may or may not be detected in an ELISA system using a soluble extract because plastic plates can hardly be sensitized with polysaccharide antigens due to the chemical nature and electrical charge of such antigens. Also, depending on the assay system, the high affinity of IgG subtypes may compete with IgG2 and IgG4 and, in some instances, block them completely. Thus, in such circumstance, an assay system of antibody capture will be able to reveal IgG2 and IgG4.

Presently, there is no commercially available antigen for the kala-azar serodiagnosis to assure the reproducibility of assay results. Recombinant antigens such as rK39 (41) or rgp63 (42) are good candidates for production by a manufacturer in order to provide a common antigen to laboratories for routine diagnosis, as well as for the study of immune response induced by different visceral *Leishmania* isolates or species.

In AVL serodiagnosis, the IFA gave high sensitivity and specificity in the detection of IgM, IgG, IgG1, IgG2, IgG3, and IgG4 antibodies (Table 1), without statistical difference. Nevertheless, IgG and IgG4 gave no false positive or negative results as did other antibody types. The IgG antibody, rather than IgG4, seems to be the choice for the diagnosis of AVL because their titers were higher and far from the cutoff titer. The IgG4 in turn tended to give some low titers close to its cutoff, despite its high GMT. Also, for public health laboratories, the IgG detection appears more suitable if the cost-benefit aspect is concerned.

The diagnostic features of the IFA in the detection of IgG4 antibodies are similar to those found for IgE-ELISA in AVL (22). It is interesting to note that both antibodies are produced by the activation of IL-4 (37,38), which is a cytokine present in active AVL (3,4). Some investigators have found in humans that parasite-specific IgG4 levels correlate with parasite-specific IgE levels (43,44).

To date, clinical improvements and the absence of parasites in clinical specimens have mostly monitored the treatment of AVL patients. An easier alternative way is to follow up patients by serology. In this work, the features of antibody decay after chemotherapy were investigated mainly in terms of GMT or units of IgG and its subtypes (Table 2), antibody conversion to negative, and type of curve disclosed by antibodies in AVL patients after treatment.

In general, the levels of IgG and its subtypes diminished after treatment and the rank order was as follows: IgG4 >

IgG3 > IgG2 > IgG > IgG1. However, if we consider the rate of antibody conversion, the order will be: IgG4 > IgG = IgG1 > IgG2 = IgG3. The study of these two features illustrates that IgG4 is an appropriate immunological marker for the evaluation of AVL patient treatment. This finding is uncommon and seems to be restricted to the Brazilian AVL, as the IgG4 is not usually detected or is detected in low levels either in the Venezuelan AVL or in the Old World VL as was discussed. In general, levels of VL antileishmanial antibodies were seen to decay after treatment; however, a number of patients have kept the same antibody levels without significant changes (12–14). Nevertheless, the GMT of antileishmanial IgG4 showed a steep decay as did the IgE antibodies, which were detected after absorbing IgG antibodies in sera from AVL patients (22).

Furthermore, we investigated the type of antibody curves disclosed by IgG and its subtypes after treatment in order to find a helpful immunological marker for the evaluation of control programs established in endemic areas for AVL. The IgG4 (Fig. 2) also proved to be a better immunological marker for monitoring population treatment than did other types of antibodies.

We also observed a significant fall in the IgG4 level at least one month post-treatment. In a period comprising 1 to 22 months following treatment, children rather than adults showed a more sensitive response to treatment as the levels of IgG4 conversion to negative were higher (Table 3) than in other types of antibodies.

The levels of IL-10 and IL-4 were decreased or disappeared after successful therapy (6,33) but in a patient who had persistent IL-4, anti-*L. chagasi* antibodies were elevated (6). Thus, the detection of IgG4 antibodies seems to mirror mostly the underlying production of IL-4 by Th2 cells.

In 26% of patients after treatment, the level of IgG4 antibody was unchanged and we can only speculate about reinfection or parasite escape that could be eliciting IgG4 antibodies, in spite of patients being considered cured by conventional criteria.

Reinforcing the latter hypothesis, latent forms of *Leishmania* have been detected inside unusual cells, as hepatocytes, in liver punctions from AVL patients after treatment (45). And, in immunocompromised patients, relapse of leishmaniasis was observed after being considered cured and having long lived outside of endemic areas (46–48). The identification of antigens or epitopes associated with antileishmanial IgG4 response is of great interest, considering that the presence of this antibody isotype reflects the lack of immunity in AVL patients. In contrast, the absence of IgG4 after chemotherapy possibly reflects the restoration of a competent immunity.

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