Rev. Inst. Med. trop. São Paulo
31(4):228-234, julho-agosto, 1989.

ANALYSIS OF THE SPECIFICITY OF HUMAN ANTIBODIES TO ANTIGENS OF LEISHMANIA BRAZILIENSIS BRAZILIENSIS

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SUMMARY

The antigenicity of promastigotes of **Leishmania braziliensis braziliensis (L. b. braziliensis)** treated with 1% sodium desoxycholate in 10 mM Tris-Hcl pH 8.2 was analysed by immunoblot using as probes sera from American cutaneous leishmaniasis (ACL), American visceral leishmaniasis (AVL), schistosomiasis, malaria and Chagas' disease. The ACL sera reacted constantly with a 60 kD band. No reactivity to this protein was observed with sera from the other diseases above mentioned indicating that the 60 kD protein may be used in serodiagnosis for ACL.

KEY WORDS: Antigens of **Leishmania braziliensis braziliensis**; Serodiagnosis of American cutaneous leishmaniasis.

INTRODUCTION

The leishmaniases comprise a complex of protozoal parasites which cause visceral, cutaneous and mucocutaneous diseases. A number of biological characteristics have been identified by which the leishmanias can be classified as different species, such as electrophorectic mobility of isoenzymes[7,15], the buoyant density of nuclear and kinetoplast DNA[5], by analysing sequence homologies in kinetoplast DNA[1] or by the use of DNA probes[3]. Also, monoclonal antibodies have been shown to differentiate species and subspecies of leishmanias which causes the ACL[13,14], greatly advancing the understanding of the antigenicity of the leishmanias, thus facilitating the identification and characterization of components potentially useful in immunoprofilatic procedures[4] or having the possibility of interfering in parasite host interactions[16].

For serodiagnosis, BADARÓ et al.[2], have described the use of a soluble antigen in micro-enzyme linked immunosorbent assay (ELISA) to antibodies in AVL which gives sensitive and specific test able to eliminate cross-reactivity with **Trypanosoma cruzi**. More recently, SANTOS et al.[17] characterized two polypeptides of 119 kD and 123 kD of **Leishmania donovani chagasi** which do not cross-react with Chagas'disease sera. In regard to ACL a 72 kD protein specific for **L.b. braziliensis**, which is recognized by sera obtained from mucocutaneous leishmaniasis, was described[11]. Most recently ULRICH et al.[19] showed that serial absorptions of the sera with well characterized strains can turn out ELISA assay, using whole formalin treated parasites, specifically reactive to certain strains. However, a well characterized **Leishmania** species specific antigen for the serodiagnosis of

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cutaneous and mucocutaneous leishmaniases which does not cross-react with **Trypanosoma cruzi** is yet to be found.

The ACL are diseases endemic in Brazil and are mainly caused by three different protozoan parasites (**Leishmania braziliensis, braziliensis, Leishmania braziliensis guyanensis** and **Leishmania mexicana amazonensis**) and their prevalence areas often overlap with areas endemic for Chagas'disease, posing a serious problem for the interpretation of immuno-diagnostic test for leishmaniasis.

The work aimed to identify antigens specifically reactive with antibody of patients with American cutaneous leishmaniasis.

MATERIALS AND METHODS

Sera

A total of 124 sera were analysed in this study and they were distributed as follows:

Group Ia 23 sera of patients with cutaneous leishmaniasis were obtained at "Instituto de Medicina Tropical de Manaus"; 9 sera of patients with cutaneous leishmaniasis and 10 sera of patients with mucocutaneous leishmaniasis were obtained at "Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo".
Group Ib 10 sera from patients with leishmaniasis caused by **L. b. braziliensis** were obtained at "Universidade Federal da Bahia" (kindly provided by Dr. Roberto Badaró).

Group II 20 sera of patients with Chagas'disease, 12 patients with AVL; 10 sera of patients with malaria; 10 sera of patients with schistosomiasis.

Group III 20 sera obtained from clinically healthy individuals (controls).

Antigen Preparation

Leishmania braziliensis braziliensis (MHOM/Br/75/M 293 strain) were cultivated in Schneider's medium supplemented with 20% fetal calf serum[8]. The parasites were harvested in late Log phase, washed five times (3.000 x g, 4°C, 15 min) in phosphate buffered saline pH 7.2 (PBS). The parasites were incubated with 1% sodium desoxycholate in 10 mM Tris-HCl pH 8.2 containing 10 μg/ml of N-Tosyl-L-phenylalanine chloromethyl ketone-TCPK (Sigma Chemical, Co) at a concentration of 10^{10} parasites/ml after vigorous vortexing, the extract was centrifuged at 40,000 x g, 4°C, 60 min. The supernatant was taken and protein content was determined by the Lowry method[12].

Micro-enzyme immunoassay (ELISA)

Fifty microliter of the antigen extract was placed in microtiter plate wells at a concentration of 20 μg/ml in carbonate buffer 0.01 M pH 9.6 and incubated overnight at 37°C. The wells were washed with PBS and blocked with 5% skim milk in PBS (SK-PBS). Then, the test sera in 100 μl amounts were added to each well in doubling dilutions and incubated for 1 h at 37°C. After, the wells were washed with 0.5% solution of PBS-Tween 20 and incubated with a 100μl of goat anti-human IgG (1/4000) or goat-antihuman IgG (1/2000) conjugated to horseradish peroxidase for 1 h at 37°C. The reaction was developed with orthophenilene diamino-benzidine-H_2O_2 (Sigma Chemical, Co), stopped with 2.5 NH_2SO_4 and wells contents were read at 490 nm in Dynatech Mini-reader (Dynatech Lab.).

Immunoblot

The antigen extract at a concentration of 500 μg/ml diluted in sample buffer containing 1% sodium dodecyl sulfate, ureia 1 M, 0.5 M DL-dithiothreitol, 10% glycerol and 0.002% bromophenol in Tris-HCl buffer pH 6.8 was placed onto a 10% polyacrylamide gel[10]. After electrophoresis, the proteins were transferred to nitrocellulose membrane (Millipore) in a transblotting chamber containing Tris (hydroxymethil amino methane) 25 mM, glycine 192 mM and 20% methanol at pH 8.3[18]. After transfering and blocking the free binding sites of the membrane with 5% SK-PBS, vertical strips were cut and the test sera diluted 1/40 in 5% SK-PBS were incubated for 2 h at room temperature. Then, they were washed with PBS and incubated with goat anti-human IgG (1/2000) or IgM (1/1000) conjugated to horseradish peroxidase for 1 h at room temperature. After washing, the reaction was

developed with diaminobenzidine-H_2O_2 (Sigma Chemical, Co).

RESULTS

ELISA

The 42 sera of patients with cutaneous or mucocutaneous leishmaniasis were titrated by ELISA using as the limit of reactivity the arithmetic mean of the absorbance obtained from 20 normal sera diluted 1/40 plus 2 standard deviations (cut off = 0.32). By the results shown in fig. 1, we observed that the titer of IgG anti-leishmanial antibodies in the sera was low. Only 2 sera in 42 samples tested were positive at 1/640 dilution. At 1/40 dilution all sera were positive and this results were confirmed by indirect immunofluorescence assay (IFA) using antigens of promastigotes of **L. b. braziliensis** fixed in 2% formalin.

The 10 sera of patients diagnosed as cutaneous leishmaniasis caused by **L. b. braziliensis** were tested at a dilution of 1/40 (Fig. 2). The reaction with peroxidase conjugated to anti-human IgG showed that 3 sera were negative and the anti-leishmanial antibodies were detected in these patients with the anti-human IgM peroxidase conjugate. Among the 10 sera tested for IgM anti-leishmanial antibodies 1 serum was negative however, this serum showed a strong reactivity when the reaction was performed with anti-human IgG peroxidase conjugate.

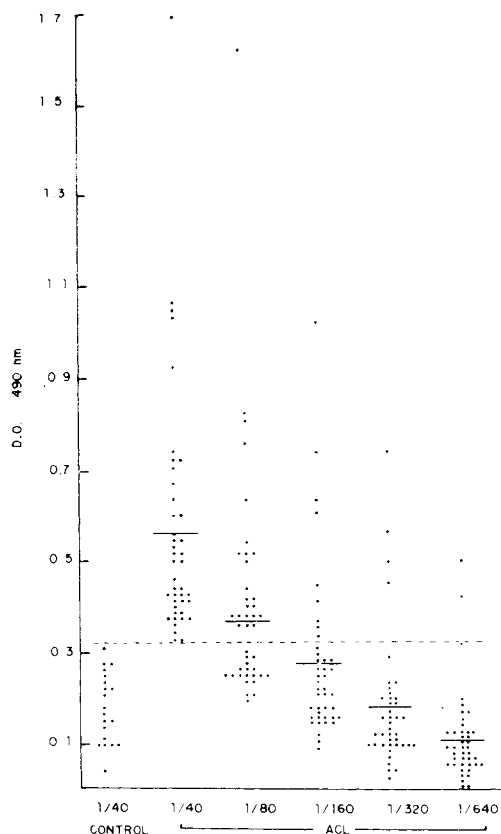

Fig. 1 — Frequency of IgG anti-leishmania antibodies titer to promastigotes of **L. b. brasiliensis** extract determined by ELISA in 42 sera of patients with American cutaneous leishmaniasis (ACL). Control: 20 sera of healthy individuals.

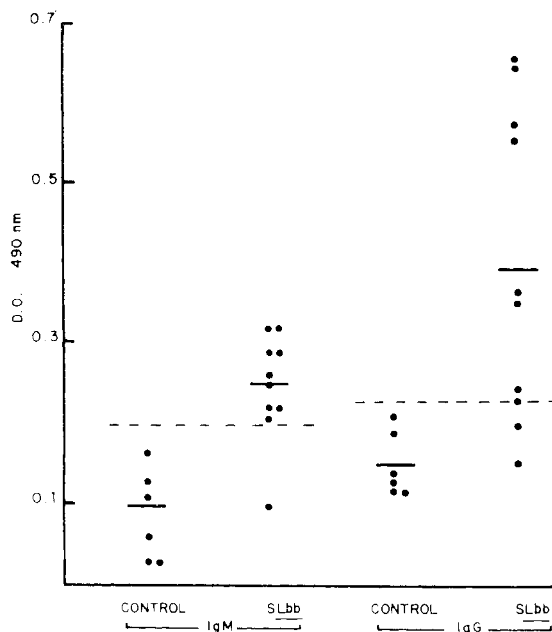

Fig. 2 — IgM and IgG anti-**Leishmania** antibodies to promastigote of **L. b. braziliensis** extract determined by ELISA in 10 sera of patients with leishmaniasis caused by **L. b. braziliensis** and in 6 sera of healthy individuals.

Immunoblot

The immunoblot analysis indicated that the 42 sera of patients with cutaneous and mucocutaneous leishmaniasis (Group Ia) showed a complex pattern of reactivity (Fig.3). However, bands of 60 kD and 67 kD were constantly reactive with these sera. Also, we observed that patients with cutaneous lesions (Fig. 3, track 1 to 6) showed lower intensity bands, with the 60 kD protein than the sera of patients with mucocutaneous lesions (Fig. 3, track 7 to 13) when detected with anti-human IgG conjugated to peroxidase. From the 10 sera of patients with leishmaniasis caused by **L. b. braziliensis** (Group Ib), 8 sera recognized the 60 kD protein when reacted with anti-human, IgM immunoglobulin (Fig. 4, track 1,2,5 to 10), with the anti-human IgG immunoglobulin 4 sera showed the 60 kD band (Fig. 4, track 6 to 9), thus only 3 sera had the IgM and IgG specific antibodies to the 60 kD protein (Fig. 4, track 6,7,8). In general, the reactivity of these sera obtained with the anti-human IgG immunoglobulin was also weak for the other proteins.

In fig. 5 it is shown the immunoblot analysis of the cross reactive antigens of **L. b.**

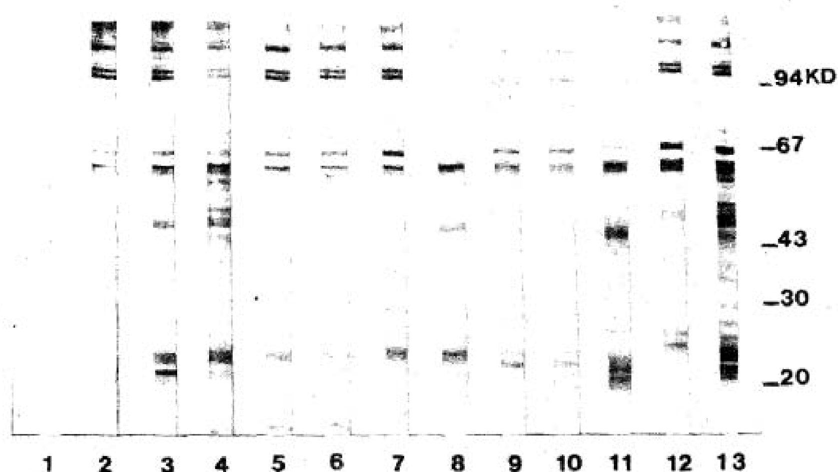

Fig. 3 — Immunoblot of sera from patients with American cutaneous leishmaniasis detected with anti-human IgG peroxidase conjugate. Track 1 to 6, sera from patients with cutaneous lesion. Track 7 to 13 sera from patients with mucocutaneous lesion.

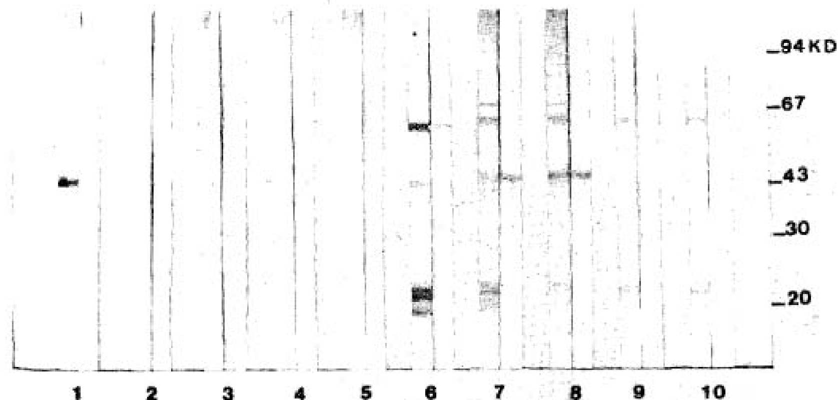

Fig. 4 — Immunoblot of sera from patients with leishmaniasis caused by **L. b. braziliensis**. The tracks on the left are blots reacted with anti-human IgM and on the right are blots reacted with anti-human IgM conjugated to peroxidase.

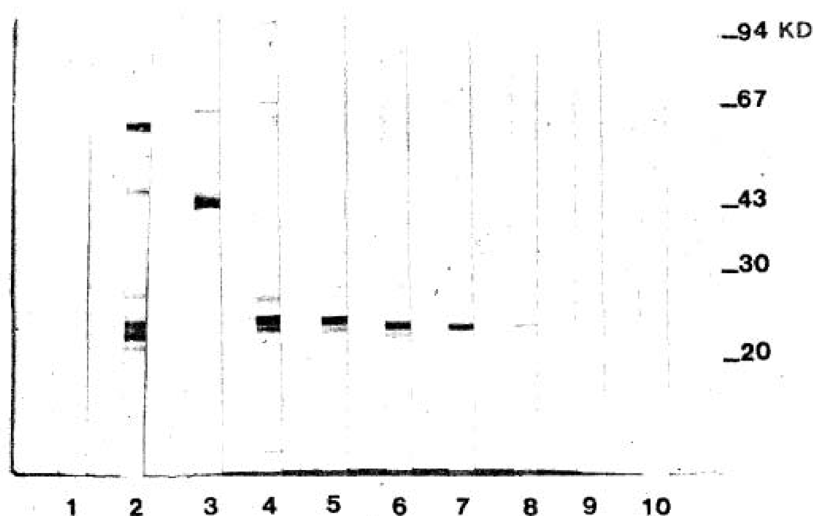

Fig. 5 — Immunoblot of pool of sera from: healthy individuals (1 negative control); patients with mucocutaneous lesion (2 positve control) visceral leishmaniasis (3 1/640): Chagas' disease (4 1/2560; 5-1/1028; 6 1/640; 7 1/320; 8 1/160); schistosomiasis (9 1/80); malaria (10 1/3200). On the right of the track number is represented the antibody titer of the pool to their causative agent. Sera were tested at a dilution of 1/40 and the reaction was detected with anti-human IgG conjugate to peroxidase.

braziliensis extracts with sera of patients bearing other parasitic diseases. In track 1 and 2 are represented the reactivity with normal sera (negative control) and with a pool of sera from patients with mucocutaneous leishmaniasis (positive control) respectively. The pool of sera from patients with AVL reacted with the 67 kD and 43 kD protein (track 3). The reactivity of sera obtained from patients with Chagas' disease which were grouped by the titer of hemagglutination test to **Trypanosoma cruzi** is shown in tracks 4 to 8. We observed that sera of chagasic patients with titer of 1/2560 recognized 6 specific bands in **L. b. braziliensis** extract but no reactivity was observed with the 60 kD protein, antibodies titer of 1/1280, 1/640 and 1/320 (tracks 5 to 7 respectively) showed 2 band around 23 kD band and the sera with titer of 1/160 only a weak band of 23 kD detected. Schistosomiasis sera showed a very faint band of 67 kD (track 9) ant the sera of patients with malaria presented no reactivity (track 10).

DISCUSSION

In this study we observed that most of the patients with ACL have low titers of specific anti-leishmanial antibodies when tested by ELISA using promastigote antigen extracted in detergent (Fig. 1) and by IFA. Also, in the well characterized sera obtained from patients which the parasites could be isolated and classified as **L. b. braziliensis,** we were able to detect specific anti-leishmanial antibodies only at 1/40 dilution of serum. Moreover, we have to search for IgM and IgG immunoglobulin in order to detect the anti-leishmanial antibodies in sera of all the patients. By these results we conclude that we are not able to use high dilution of serum to overcome the problem of cross reactivity among ACL, AVL and specially with Chagas'disease sera. Therefore, we analysed the antigenicity of promastigotes of **L. b. braziliensis** extracted in detergent by immunoblot procedure. The ACL sera showed a complex pattern of reactivity with this extract. However, we observed that all 42 sera with chronic cutaneous or mucocutaneous lesions recognized a 60 kD and a 67 kD protein (Fig. 3). In the group of patients in which the parasites could be isolated, 2 out of 10 sera failed to react with the 60 kD protein (Fig. 4). This is not a surprising result since a more specific serodiagnosis method usually leads to a lower sensitivity in the test It is likely that the 60 kD antigen will not be a subspecies specific

antigen since the 23 sera obtained in Amazon region where the presence of **L. b. braziliensis** is rare[6] also recognized the 60 kD protein. These results suggests that the 60 kD protein may be useful in the diagnosis of ACL since sera from patients with the other diseases above mentioned did not recognized the 60 kD band (Fig. 5).

We were not able to detect reactivity with the 72 kD protein which has been described by LEGRAND et al.[11] with our antigen extract. This may be due to difference in the antigen preparation as well as in the **L. b. braziliensis** strain used.

Species and subspecies specific antigens have been characterized by hiperimmunesera[9] or monoclonal antibodies[13,14]. However, methods for serodiagnosis showing these specificities have not been described. The failure to find a more specific antigen could be due to the source of antigen utilized in the tests. In general, the studies have been done with promastigotes obtained in culture which are not the stage that grow in the vertebrate host. Thus, it is possible that the species specific antigens recognized by human sera are epitopes present in amastigotes.

RESUMO

Análise da especificidade de anticorpos humanos a antígenos de Leishmania braziliensis braziliensis

A antigenicidade de promastigotas de **Leishmania braziliensis braziliensis** tratadas com desoxicolato de sódio 1% tampão Tris-HCl 10 mM pH 8.2 foi determinada por immunoblot usando soros de pacientes com leishmaniose cutânea e mucocutânea, leishmaniose visceral, esquistossomose, malária e doença de Chagas. Os soros de pacientes com leishmaniose cutânea e mucocutânea apresentaram reação positiva com uma banda de 60 kD. Não se observou reatividade para esta fração em soros de pacientes com outras doenças parasitárias acima mencionadas, indicando que esta pode ser utilizada no sorodiagnóstico de leishmaniose tegumentar.

ACKNOWLEDGEMENTS

The authors greatefully acknowledge Dr. Roberto Badaró for kindly provinding sera of patients with specific diagnosis of leishmaniasis caused by **L. b. braziliensis**. Paulo de Oliveira and Rivaldo de Souza Cardoso for the excellent Technical assistance and Ceci Corrêa Godoi de Angelis and Eunice Bonfim Pinto for editorial assistance.

This work was partially supported by Fundação de Amparo à Pesquisa do Estado de São Paulo - 86/2389, by the UNDP/WORLD/BANK/WHO Special Programme for Research and Training in Tropical Diseases (ID - 870 250); by Laboratório de Investigação Médica - HC-FMUSP - (LIM-48); and by FINEP (43.88.0689/00).

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Recebido para publicação em 1/2/1989.