JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1991, p. 595–599
0095-1137/91/030595-05$02.00/0

Vol. 29, No. 3

Antigenic Specificity of the 72-Kilodalton Major Surface Glycoprotein of *Leishmania braziliensis braziliensis*

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Received 13 March 1990/Accepted 10 December 1990

We examined the expression and the antigenicity of the major surface polypeptides of *Leishmania braziliensis braziliensis* and *Leishmania donovani chagasi*, parasites which commonly coexist in the same endemic areas of Bolivia. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein profiles from surface-iodinated promastigotes showed the presence of a unique iodinatable polypeptide of 72 kDa on the *L. b. braziliensis* surface and of two major components of 65 and 50 kDa exposed at the surface of *L. d. chagasi*. Comparison of the peptide digestion profiles of the major iodinated polypeptides of both strains showed no similarity between the maps of the 72- and the 65-kDa polypeptides of *L. b. braziliensis* and *L. d. chagasi*, respectively. Immunoprecipitation of surface-labeled *L. b. braziliensis* Nonidet P-40 extracts with 35 serum specimens obtained from Bolivian patients with cutaneous and mucocutaneous leishmaniasis showed that all serum specimens recognized predominantly the 72-kDa antigen and high-molecular-mass proteins in some cases. The recognition patterns were independent of the geographical origin of the patient, the type of lesion, and the serum antibody titer. Serum specimens from children with visceral leishmaniasis did not precipitate the *L. b. braziliensis* 72-kDa antigen. Hamster hyperimmune serum against *L. b. braziliensis* also recognized the 72-kDa surface antigen. However, this recognition was inhibited in the presence of the homologous nonlabeled antigen but not in the presence of heterologous (*L. d. chagasi* and *Trypanosoma cruzi*) antigens. The specific recognition of the 72-kDa surface antigen in both natural and experimental *L. b. braziliensis* infections suggests that this antigen could be a good candidate for use in the differential immunodiagnosis and prognosis of the disease.

Human leishmaniasis includes a group of diseases with different clinical manifestations: cutaneous, mucocutaneous, and visceral. These diseases occur throughout the world and, in endemic tropical areas, represent a great public health problem (6, 20). The etiological agents of leishmaniasis include many complex and epidemiologically diverse species from the genus *Leishmania* (16).

In South America, particularly in Bolivia, the species of *Leishmania* present are mainly *Leishmania braziliensis braziliensis* and *Leishmania donovani chagasi*; they are the causative agents of human mucocutaneous and visceral leishmaniasis, respectively (19). Both types of leishmaniasis are frequently endemic in the same areas of the country (i.e., the Yungas valleys, Department of La Paz), rendering epidemiological studies difficult (7, 8).

During the past few years, particular emphasis has been given to characterization of antigenic components on the promastigote surface as a tool in obtaining specific *Leishmania* antigens for parasite identification as well as for specific diagnosis and immunoprophylaxis. Several promastigote cell surface antigens have been well characterized. Among them, the promastigote surface protease, a predominant surface glycoprotein (gp63) with an apparent molecular mass of 63 to 65 kDa, was identified as a highly conserved and cross-reactive antigen shared by most of the *Leishmania* species tested so far (1–3, 5, 10, 11, 18). However, Legrand and coworkers (17) recently demonstrated the presence of a polypeptide of 72 kDa as the predominant surface antigen in 12 different isolates of *L. b. braziliensis* (10 local Bolivian strains isolated from patients or sandflies and 2 Brazilian reference strains) instead of the gp63-gp65 major surface antigens present in other Old and New World *Leishmania* species, including *L. d. chagasi*.

Based on the findings described above, we performed a comparative study of the major surface polypeptides of *L. b. braziliensis* and *L. d. chagasi* and analyzed their antigenic properties in experimental and natural infections.

MATERIALS AND METHODS

Parasites and culture conditions. Promastigotes of an *L. b. braziliensis* reference strain (MHOM/BR/75/M-2903) and an *L. d. chagasi* reference strain (MHOM/BR/74/M-2682) were grown at 28°C in Schneider *Drosophila* medium (GIBCO Bio-Cult, Paisley, United Kingdom) supplemented with 15% (vol/vol) heat-inactivated fetal bovine serum (Serva, Heidelberg, Federal Republic of Germany).

Human and hamster sera. Serum specimens from 35 Bolivian patients with cutaneous and mucocutaneous leishmaniasis were collected in our laboratory. All the patients were clinically diagnosed and exhibited a positive skin test reaction, and their sera showed positive serology for *L. b. braziliensis*, as detected by immunofluorescence (in most cases, the antibody titers were very low, in the range of 1/20 to 1/40, and in some cases they were negative) (9). In some cases the parasite was isolated from the lesions and characterized as *L. b. braziliensis* by isoenzyme electrophoresis (7). In addition, serum specimens from two Bolivian patients

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with visceral leishmaniasis infected with *L. d. chagasi* were used (8).

Hamster hyperimmune antiserum against *L. b. braziliensis* was collected 1 year after footpad inoculation of hamsters with viable promastigotes of the strain MHOM/BO/84/LPZ-688.

Surface iodination and detergent solubilization of proteins. Late-log-phase promastigotes (2×10^8) were surface iodinated with 200 μCi of carrier-free $Na^{125}I$ (ORIS, Gif-sur-Yvette, France) and Iodo-Gen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril; Sigma) as described by Legrand et al. (17). After iodination and washing of the unbound ^{125}I, the cell pellets were extracted for 2 h at 4°C with 200 μl of 0.5% Nonidet P-40 in 20 mM Tris (pH 7.4)–300 mM NaCl containing aprotinin (100 U/ml). The detergent-insoluble material was removed by centrifugation, and lysates were stored at −70°C until use.

Gel electrophoresis and autoradiography. Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on slab gels containing 7.5% polyacrylamide (15). After staining of the proteins with Coomassie blue, the gels were dried and autoradiographed at −70°C with X-OPMAT AR film (Eastman Kodak, Rochester, N.Y.) in conjunction with Cronex intensifying screens (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.). Bands in the autoradiograms were quantified by densitometry (Hoefer Scientific Instruments, San Francisco, Calif.).

Peptide mapping. Partial proteolytic maps of the major surface-iodinated proteins were performed as described by Cleveland et al. (4). Proteins were separated by SDS-PAGE on 7.5% polyacrylamide gels. After electrophoresis, gel pieces containing the major iodinated proteins were excised and run on a second 12.5% polyacrylamide gel with sample buffer containing *Staphylococcus aureus* V8 protease (1 to 50 μg/ml; Boehringer, Mannheim, Federal Republic of Germany). The products of partial proteolysis were revealed by autoradiography.

Immunoprecipitation and immunocompetition. Immunoprecipitation was carried out by the method of Kessler (14). A detergent extract of labeled promastigotes (10 μl; 10^7 cells) was diluted in 450 μl of 10 mM Tris (pH 8)–150 mM NaCl–2 mM EDTA–0.5% (vol/vol) Nonidet P-40–100 U of aprotinin per ml and incubated with 50 μl of serum for 1 h at room temperature with constant agitation. Immune complexes were absorbed for 1 h with 5 mg of protein A–Sepharose 4B-CL (Pharmacia, Uppsala, Sweden) and suspended in the buffer described above. Absorbed antigens were solubilized in 20 μl of sample buffer and stored at −20°C for SDS-PAGE.

For immunocompetition experiments, 10 μl of the labeled extract was incubated with the serum in the presence of 50 μl of homologous or heterologous nonlabeled antigen extract and processed for absorption with protein A–Sepharose as described above.

RESULTS

Identification and peptide mapping analysis of surface-labeled *L. b. braziliensis* and *L. d. chagasi* polypeptides. *L. b. braziliensis* and *L. d. chagasi* promastigotes were surface iodinated and extracted with Nonidet P-40, and the proteins were resolved by SDS-PAGE. The autoradiographic profiles revealed a unique surface-labeled polypeptide of 72 kDa in the *L. b. braziliensis* extract (Fig. 1, lane 1) and two major labeled proteins of 65 and 50 kDa in the *L. d. chagasi* extract (Fig. 1, lane 2).

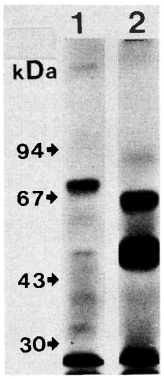

FIG. 1. Autoradiographic patterns of iodinated surface proteins of *L. b. braziliensis* and *L. d. chagasi* promastigotes. Samples of radioiodinated *L. b. braziliensis* and *L. d. chagasi* were analyzed by SDS-PAGE followed by autoradiography. A total of 2×10^7 promastigotes was loaded into each slot. Lane 1, *L. b. braziliensis*; lane 2, *L. d. chagasi*. The arrows indicate the migration of the protein standards.

In order to determine a structural relationship between the 72- and 65-kDa proteins of *L. b. braziliensis* and *L. d. chagasi*, peptide mapping analysis of both polypeptides was carried out (Fig. 2). Peptide digestion profiles of the 72- and 65-kDa proteins with the V8 protease of *S. aureus* showed that both proteins were sensitive to digestion by the enzyme; however, the overall patterns obtained were significantly different. No similarity was found in the apparent molecular weights of any of the partial proteolysis products. Distinct peptide patterns were also obtained upon cleavage of the proteins at tryptophan by using *N*-chlorosuccinimide (to be published elsewhere). These results might indicate that the 72- and 65-kDa polypeptides of *L. b. braziliensis* and *L. d. chagasi* are not structurally related.

Antigenic recognition of major surface polypeptides in experimental *L. b. braziliensis* infection. Hamster hyperimmune serum to *L. b. braziliensis* was used to precipitate labeled *L. b. braziliensis* and *L. d. chagasi* surface antigens from parasite extracts. SDS-PAGE immunoprecipitation patterns showed that the hamster serum recognized strongly and specifically the 72-kDa protein expressed on the surface

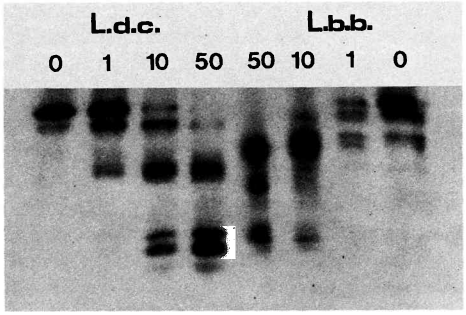

FIG. 2. Peptide mapping of the major *L. b. braziliensis* (L.b.b.) and *L. d. chagasi* (L.d.c.) surface polypeptides. Labeled 72- and 65-kDa polypeptides were excised from the gel shown in Fig. 1 and submitted to proteolytic digestion with different concentrations of *S. aureus* V8 protease (numbers above the lanes indicate the protease concentration, in micrograms per milliliter). The products of partial proteolysis were separated by SDS-PAGE and were autoradiographed.

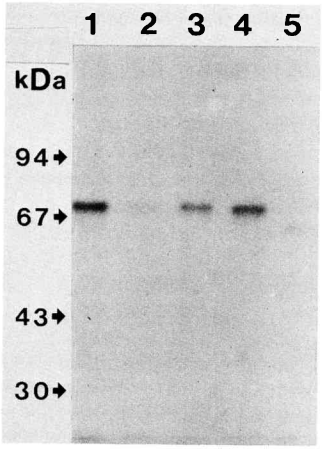

FIG. 3. Immunoprecipitation of *L. b. braziliensis* and *L. d. chagasi* surface proteins with hamster hyperimmune serum and immunocompetition. Nonidet P-40 extracts from surface-iodinated promastigotes were reacted with serum from an *L. b. braziliensis*-infected hamster, and the precipitated proteins were resolved by SDS-PAGE and autoradiographed. Lanes 1 and 5, precipitation patterns of radioiodinated *L. b. braziliensis* and *L. d. chagasi* extracts, respectively; lanes 2 to 4, immunocompetition-precipitation of radioiodinated *L. b. braziliensis* extract with the anti-*L. b. braziliensis* serum in the presence of either homologous *L. b. braziliensis* (lane 2) or heterologous *L. d. chagasi* (lane 3) and *T. cruzi* (lane 4) nonlabeled extracts.

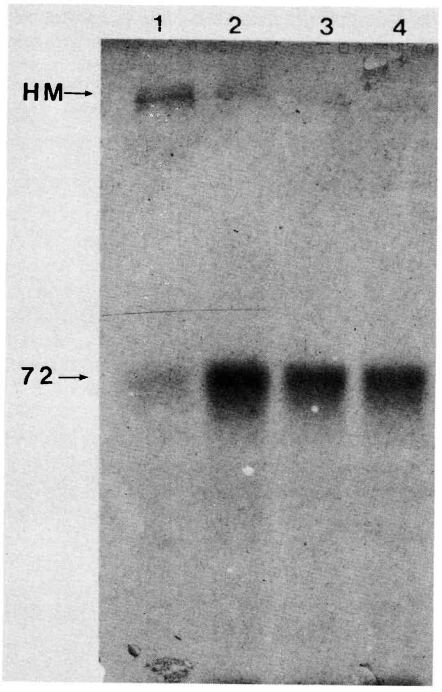

FIG. 5. Antigenic recognition of *L. b. braziliensis* surface proteins during the course of infection of a single patient. Extracts from surface-iodinated *L. b. braziliensis* were immunoprecipitated with sera from a patient collected at different times during the course of an *L. b. braziliensis* infection. Lane 1, Immunoprecipitation with serum collected at the beginning of the infection (primary lesion in November 1984); lane 2, serum collected after Glucantime treatment (March 1985); lane 3, serum collected 1 year after treatment; lane 4, serum collected at the beginning of a mucous lesion (February 1988). HM, High-molecular-mass antigens; 72, the 72-kDa major surface antigen.

of *L. b. braziliensis* promastigotes (Fig. 3, lane 1). However, hamster serum recognized very poorly (less than 20%) the 65-kDa surface polypeptide of *L. d. chagasi* (Fig. 3, lane 5).

In order to assess the degree of cross-reactivity between the major surface antigens of these two *Leishmania* subspecies, immunocompetition experiments were carried out. Immunoprecipitation of labeled *L. b. braziliensis* Nonidet P-40 extracts with the hamster serum was performed in the presence of nonlabeled *L. b. braziliensis* or *L. d. chagasi* antigen extracts. A *Trypanosoma cruzi* antigen extract was also tested in the competition experiments because Chagas' disease is frequently coendemic with mucocutaneous and visceral leishmaniasis in Bolivia. Up to 92% inhibition was obtained when the precipitation was carried out in the presence of the homologous *L. b. braziliensis* antigen (Fig. 3, lane 2). However, only a slight inhibition was observed when heterologous *L. d. chagasi* and *T. cruzi* antigens were used (24 and 13%, respectively) (Fig. 3, lanes 3 and 4). The inability of the heterologous antigens to fully inhibit the precipitation reaction suggested that there is a low degree of cross-reactivity between them and the 72-kDa surface antigen of *L. b. braziliensis*.

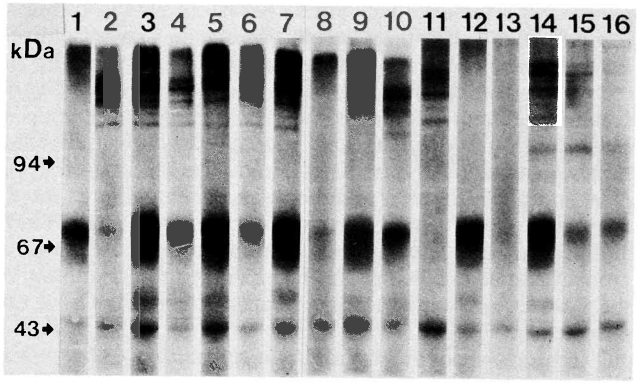

FIG. 4. Antigenic recognition of *L. b. braziliensis* surface proteins by sera from Bolivian patients with cutaneous and mucocutaneous leishmaniasis. Immunoprecipitation patterns of iodinated *L. b. braziliensis* surface proteins precipitated with different sera after SDS-PAGE and autoradiography. Lanes 1 to 12 and 14 to 16, *L. b. braziliensis* extracts; lane 13, *L. d. chagasi* extract. The descriptions of the different serum specimens are given in Table 1 (serum specimen numbers in Table 1 correspond to lane numbers).

Antigenic recognition of the surface antigens in natural *L. b. braziliensis* infections. Serum specimens from 35 *L. b. braziliensis*-infected patients presenting cutaneous or mucous lesions and originating from different endemic areas of Bolivia were chosen arbitrarily. These serum specimens were used to immunoprecipitate iodinated *L. b. braziliensis* extracts, and the results for 16 representative samples are shown in Fig. 4. Up to 95% of the serum specimens recognized the 72-kDa antigen of *L. b. braziliensis*, and some of them revealed additional high-molecular-mass components (lanes 1 to 12 and 14 to 16). Serum from a patient with visceral leishmaniasis did not recognize any of the labeled components on the *L. b. braziliensis* surface (Fig. 4, lane 13).

No apparent correlation could be found between the different patterns of recognition obtained, on the one hand, and the geographical origin of the patient, the clinical man-

TABLE 1. Recognition of *L. b. braziliensis* antigens by sera from Bolivian patients with cutaneous and mucocutaneous leishmaniasis

Serum specimen no.	Geographical area	Type of leishmaniasis	Antibody titer	Antigen recognition	
				72-kDa antigen	High-molecular-mass antigen
1	San Borja (Beni)	Cutaneous	1/40	++	−
2	San Borja (Beni)	Cutaneous	1/40	+	+++
3	Alto Beni	Cutaneous	1/20	+++	+++
4	San Borja (Beni)	Cutaneous	1/40	++	++
5	Alto Beni	Cutaneous	1/40	+++	+++
6	Chapare	Cutaneous	1/20	+	+++
7	Ixiamas (Alto Beni)	Cutaneous	Negative	+++	+++
8	Chulumani (Yungas)	Mucocutaneous	1/20	+	−
9	Chapare	Cutaneous[a]	1/80	+++	+++
10	Chapare	Cutaneous[a]	1/40	++	++
11	Alto Beni	Cutaneous	1/40	−	+++
12	Caranavi (Yungas)	Mucocutaneous	1/40	+++	−
13	Yungas	Visceral	1/80	−	−
14	Coroico (Yungas)	Mucocutaneous	1/80	+++	+++
15	Quillabamba (Peru)	Mucocutaneous	1/40	+	+
16	Yungas	Mucocutaneous	1/80	+	−

[a] Patients with associated Chagas' disease.

ifestation of the disease, and the antibody titer of the sera, on the other (Table 1). Nevertheless, a correlation between the immune recognition of the 72-kDa antigen and the progress of the infection could be evaluated. This was carried out by using sera from a patient infected with *L. b. braziliensis* who was monitored from the beginning of the infection until the appearance of the mucous lesion (Glucantime [meglumine antimoniate] was used to treat the patient, but it did not cure the patient; there was a low antibody titer [1/20]). SDS-PAGE immunoprecipitation profiles of *L. b. braziliensis* extract reacted with the patient sera, which were collected at various stages of the disease (Fig. 5), showed that at the beginning of the infection, at the time of the appearance of the primary lesion, the serum recognized mostly the high-molecular-mass components and, to some extent, the 72-kDa antigen (Fig. 5, lane 1). With the progression of the infection (Fig. 5, lanes 2 and 3), recognition of the 72-kDa antigen increased, while, inversely, that of the high-molecular-mass antigen decreased until the mucous lesion became manifested (Fig. 5, lane 4).

DISCUSSION

The expression of a 72-kDa antigen on the surface of *L. b. braziliensis* promastigotes was previously reported by Legrand and coworkers (17) in a comparative study with 12 different *L. b. braziliensis* isolates. Other New World *Leishmania* species belonging to the same *L. b. braziliensis* complex (*L. braziliensis panamensis* and *L. braziliensis guyanensis*) or to other complexes (*L. m. amazonensis* and *L. d. chagasi*) were shown to express a 63- to 65-kDa polypeptide on their surfaces, as demonstrated by surface radioiodination and immunoprecipitation protein profiles (2).

In the present study we extended the studies described above and structurally compared, by peptide mapping analysis, the major 72- and 65-kDa polypeptides expressed on the surface of *L. b. braziliensis* and *L. d. chagasi*, respectively. Our results demonstrated that these polypeptides not only differ in their molecular masses but are structurally different, inasmuch as no homology could be observed in their peptide digestion profiles. This result is in disagreement with those previously reported by other investigators (5, 10) regarding the structural homology of the major surface antigens from various *Leishmania* strains, including *L. b. braziliensis*. Further sequencing of these proteins is required in order to fully prove structural differences.

In the same work, Legrand and coworkers (17) showed that the *L. b. braziliensis* 72-kDa major surface antigen was not recognized by sera from patients with visceral leishmaniasis or Chagas' disease, although recognition of this antigen was achieved by serum from a patient with mucocutaneous leishmaniasis and by serum from a hamster infected with *L. b. braziliensis*.

In this study we demonstrated that specific recognition of the *L. b. braziliensis* 72-kDa antigen is a property of the majority of the serum specimens from 35 Bolivian patients naturally infected with *L. b. braziliensis*. Moreover, the structural differences we observed in the present study correlated with the antigenic recognition obtained both by sera from hamsters with experimental infections and by patient sera. In either case, the 72-kDa antigen was demonstrated to be highly immunogenic and specifically recognized by the serum specimens from hamsters experimentally infected and from patients with *L. b. braziliensis* but not by the serum specimens from patients with visceral leishmaniasis. However, under the same experimental conditions the hamster sera failed to recognize the 65-kDa antigen of *L. d. chagasi*. Those results, together with the inability of the *L. d. chagasi* and *T. cruzi* antigens to fully inhibit the 72-kDa antigen-antibody recognition, demonstrate a low level of cross-reactivity and might suggest an antigenic specificity of the 72-kDa polypeptide expressed on the surface of *L. b. braziliensis*.

The heterogeneity in *Leishmania* cell surface antigens has been postulated by several groups of investigators (2, 12, 13). The need to study those antigens in more depth has been stressed, since their expression may differ among the various *Leishmania* species. This is particularly true if we take into consideration the diversity of *Leishmania* strains, the variety of vectors, the geographical habitats, the reservoir hosts, the different tissue tropisms, and the various clinical manifestations of the disease. Even though the existence of

common antigens among *Leishmania* species could help in designing immunoprophylactic protocols against the disease, the characterization of species- and subspecies-specific antigens such as the 72-kDa antigen of *L. b. braziliensis* may be important for both taxonomic studies and practical identification techniques. Such a specific antigen can also represent a good candidate for the differential diagnosis and prognosis of mucocutaneous leishmaniasis in endemic areas where various types of *Leishmania* and other protozoan parasites coexist.

ACKNOWLEDGMENTS

This work was partially supported by grants from the Ministère des Affaires Etrangères (Paris, France), the United Nations Development Programme/World Bank/World Health Organization Special Program for Research and Training in Tropical Diseases, and the Rockefeller Foundation.

We acknowledge A. G. Hernandez for advice and fruitful discussions and Sergio Mollinedo for providing the sera from Bolivian patients.

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