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Identification of cytoplasmic soluble antigens related to the major surface antigens of *Leishmania braziliensis braziliensis* and *L. donovani chagasi*

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Abstract. This study describes the identification of aqueous-soluble antigens in *Leishmania* promastigotes immunologically and biochemically closely related to the major surface antigen. Proteins from surface-iodinated *L. braziliensis braziliensis* and *L. donovani chagasi* promastigotes, extracted and separated by partitioning in the detergent Triton X-114, were analyzed. Immunoblotting of the extracted proteins, using homologous antisera, showed recognition of a 72-kDa labeled, amphiphilic antigen of *L. b. braziliensis* and a 65-kDa surface antigen of *L. d. chagasi*. The respective homologous sera also recognized non-labeled hydrophilic antigens, similar in their apparent molecular weights to the major surface antigens. The amphiphilic and hydrophilic antigens of each species were found to share common antigenic determinants, inasmuch as monospecific antibodies that recognized the amphiphilic protein reacted with the hydrophilic antigen. Structural homology was also obtained in the peptide-digestion profiles of the amphiphilic and the respective hydrophilic major antigens. Zymogram assay showed that both amphibilic and hydrophilic fractions displayed proteolytic activity that could be directly attributed to the major *L. b. braziliensis* and *L. d. chagasi* antigens. The hydrophilic antigens found in this study are probably not hydrolytic products of the surface antigens and occur in large quantities in the promastigote cytosol.

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Many studies on surface antigens of different *Leishmania* species from the Old and New World assert the presence of a major surface antigen with an apparent molecular weight of 63–65 kDa in promastigotes of *L. tropica* (Handman and Curtis 1982; Gardiner and Dwyer 1983), *L. major* (Bouvier et al. 1985), *L. donovani* (Lepay et al. 1983; Lemesre et al. 1985), *L. mexicana* (Chang et al. 1986) and *L. brasiliensis* (Misle et al. 1985) and that of a 72-kDa major surface antigen for the sub-species *L. b. braziliensis* (Legrand et al. 1987). Except for the latter, these glycoproteins exhibit cross-reactivity with heterologous sera and share a common primary structure in most *Leishmania* species tested (Etges et al. 1985; Colomer-Gould et al. 1985). Recently, proteolytic activity related to the major surface antigen in promastigotes was also reported (Etges et al. 1986; Bordier 1987; Bouvier et al. 1987; Chaudhuri and Chang 1988).

Several methods have been used to purify the major surface antigen of *Leishmania*, including affinity chromatography with specific monoclonal antibodies (Chang and Chang 1986) or concanavalin A (Russell and Wilhelm 1986) or phase partitioning with the detergent Triton X-114 (Bouvier et al. 1985). Using the latter technique with *L. major* promastigotes, Bouvier and co-workers identified a soluble form of the surface antigen generated in vitro during its purification, probably by the hydrolysis of a lipid-containing myristyl residue that anchored the protein in the membrane.

In this work we report the identification of cytoplasmic soluble antigens that are biochemically and immunologically closely related to the major surface antigens of *L. b. braziliensis* and *L. d. chagasi* promastigotes. The results suggest that these soluble antigens are not generated in vitro during the Triton X-114 extraction, but rather are present

in significant amounts in the promastigote cytoplasm. The biological nature and the biochemical implications of these antigens are discussed.

Materials and methods

Parasites and culture conditions. Promastigotes of *L. b. braziliensis* (reference strain from Brazil MHOM/BR/75/M-2903) and *L. d. chagasi* (reference strain MHOM/BR/74/M-2682) were grown at 28° C in Schneider *Drosophila* medium (Gibco Bio-Cult, Paisley, UK) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Serva, Heidelberg, FRG).

Human sera. Sera from Bolivian patients with mucocutaneous and visceral leishmaniasis were collected in our laboratory. Clinically diagnosed cases were serologically and parasitologically confirmed.

Iodination and Triton-X114 solubilization. A total of 2×10^8 late-log-phase promastigotes were surface-radioiodinated with $Na^{125}I$ (Oris, Gif Sur Yvette, France) and Iodo-Gen (1,3,4,6-tetrachloro-3,6-diphenylglycoluril from Sigma, Saint Louis, USA) as described by Legrand et al. (1987). Promastigote proteins were extracted with the detergent Triton X-114 (Sigma) according to Etges et al. (1985). In all, 0.2 ml 2% (v/v) precondensed Triton X-114 (Bordier 1981) in TBS (10 m*M* TRIS, 150 m*M* NaCl; pH 7.4) containing 100 units/ml aprotinine (Sigma) was used for each 2×10^8 promastigotes. After extraction at 0° C for 10 min, the insoluble material was precipiated by centrifugation at 30000 *g* for 45 min at 4° C and the supernatant was applied to a 6% (w/v) sucrose cushion, incubated for 3 min at 37° C and centrifuged at *1000 g* for *10* min at room temperature to sediment the detergent aggregates through the cushion. Aqueous and detergent phases were recovered above and below the cushion, respectively. Both phases were adjusted to the same final Triton concentration (2%) by mixing with the reciprocal phases obtained from a phase separation without cells.

Iodination of aqueous-phase proteins was carried out as described above after Triton X-114 extraction of 2×10^8 non-labeled promastigotes. Proteins were desalted on a PD-10 column (Pharmacia Fine Chemicals, Uppsala, Sweden), equilibrated with phosphate-buffered solution (PBS) supplemented with 0.3 *M* NaCl (PBS-NaCl) containing 2 mg/ml bovine serum albumin (BSA) (Sigma).

Gel electrophoresis, immunoblotting and autoradiography. Extracts were resuspended in sample buffer [4% (w/v) sodium dodecyl sulfate (SDS), 2.5% (v/v) mercaptoethanol] and heated for 10 min at 80° C. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on slab gels containing 7.5% polyacrylamide (Laemmli 1970) and stained with Coomassie blue.

For immunoblotting, extracted proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose paper (Battliger et al. 1982). After saturation in TBS containing milk (5%) and Tween 20 (0.05% v/v), the nitrocellulose was incubated with the respective human anti-sera (Bolivian patients). Identification of reactive antigens was done with anti-human antisera linked to peroxidase [IgG (H + L); Diagnostics Pasteur, Marnes-la-Coquette, France] using 3,3′ diaminobenzidine (Sigma) as the peroxidase-specific substrate. Bands on the nitrocellulose were quantified by densitometry (Hoefer Scientific Instruments, San Francisco, USA). For the detection of radioactively labeled proteins, dried gels or blots were autoradiographed at −70° C on X-OMAT AR film (Eastman Kodak, Rochester, NY, USA) and Cronex intensifying screens (Dupont de Nemours, Wilmington, USA).

Elution of specific antibodies from nitrocellulose. Preparative gels containing the detergent-phase proteins were transferred to nitrocellulose and incubated with the respective antisera. After incubation, a strip of the nitrocellulose was cut out and immunodetected as described above to localize the position of the major surface antigen. The corresponding band was excised from the preparative nitrocellulose and the bound antibodies were eluted by three successive 10-min incubations in glycine buffer (pH 2.8) (0.2 *M* glycine, pH 2.8; 150 m*M* NaCl), neutralized with milk-TBS buffer (pH 8) and incubated with a second blot containing aqueous-phase proteins. The reactive antigens were identified as described above.

Peptide mapping. Partial proteolytic mapping of the iodinated amphiphilic and hydrophilic antigens was carried out according to Cleveland et al. (1977). After SDS-PAGE (7.5% polyacrylamide) of extracted proteins, gel pieces containing the major iodinated proteins were excised and coelectrophoresed on a second 12.5% polyacrylamide gel with sample buffer containing *Staphylococcus aureus* V8 protease (1–50 µg/ml) (Boehringer, Mannheim, FRG). The gels were dried and submitted to autoradiography.

Protease activity in gels. Zymograms were obtained by SDS-PAGE but with the addition of 240 µg/ml bovine fibrinogen (Schwarz/Mann, USA) to the running gel (Bouvier et al. 1987). In this case, samples were neither heated nor reduced. After the electrophoresis, the gels were washed three times for 30 min in TBS (pH 8) and incubated overnight in the same buffer at 37° C. Gels were stained and destained as described above and then autoradiographed.

Results

Identification of antigens from surface-iodinated promastigotes

Proteins from surface-labeled *L. b. braziliensis* and *L. d. chagasi* promastigotes were extracted with Triton X-114 and detergent and aqueous phases were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose paper. Blots from *L. b. braziliensis* proteins were incubated with the serum of a mucocutaneous patient (Fig. 1a; lanes 1, 3), whereas blots from *L. d. chagasi* proteins were incubated with the serum of a child with visceral leishmaniasis (Fig. 1b; lanes 1, 3).

Antibodies present in the serum of the mucocutaneous patient recognized a 72-kDa protein of *L. b. braziliensis* extracted in the detergent phase (Fig. 1a; lane 1), which is the major surface-labeled component, as shown by the respective autoradiogram (Fig. 1a; lane 2). This result is in agreement with those previously reported by Legrand et al. (1987) regarding the presence of a major surface antigen of 72 kDa in NP-40 extracts of *L. b. braziliensis* promastigotes. Moreover, the

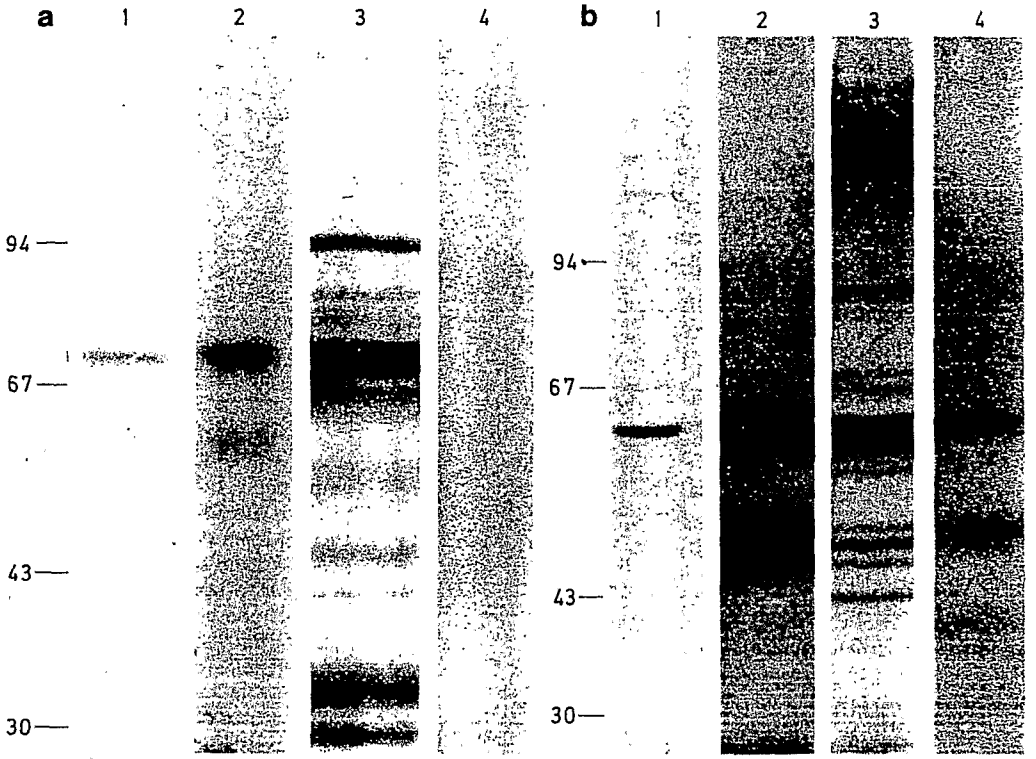

Fig. 1a, b. Immunological reactivity of *L. b. braziliensis* and *L. d. chagasi* detergent- and aqueous-soluble proteins. Surface-iodinated promastigotes were extracted by Triton X-114 and proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose paper. The amount of protein loaded in the gel was equivalent to 2×10^7 parasites/slot. The blots were incubated with the homologous human antisera, revealed and autoradiographed. **a** *L. b. braziliensis* reacted with sera from a mucocutaneous patient. **b** *L. d. chagasi* reacted with sera from a patient with visceral leishmaniasis. *Lanes 1, 3:* blots from the detergent- and aqueous-soluble proteins, respectively. *Lanes 2, 4:* autoradiograms from blots 1 and 3, respectively. *Arrows* represent the migration of the protein standards

mucocutaneous serum recognized a number of proteins extracted in the aqueous phase but, strongly and predominantly, a protein of the same apparent molecular weight of 72 kDa (Fig. 1a; lane 3). No radioactivity was found in the respective autoradiogram (Fig. 1a; lane 4). In the case of *L. d. chagasi*, two major, labeled components of 65 and 50 kDa were observed in the detergent phase, but only the first one was recognized by the visceral sera (Fig. 1b; lanes 1, 2). This serum also strongly recognized a hydrophilic, non-labeled, 65-kDa antigen in the aqueous phase (Fig. 1b; lanes 3, 4).

As measured by densitometric analysis, the quantitative ratio of the detergent-soluble to the respective aqueous-soluble major antigens was at least 1:6, suggesting that the major hydrophilic antigens occur in significant amounts in the promastigote cytoplasm.

Immunological cross-reactivity of membrane and soluble antigens

Since the respective polyvalent human sera recognized major promastigote membrane and soluble antigens with similar apparent molecular weights, their immunological relationship was further evaluated. For each parasite species, preparative immunoblotting was carried out using detergent-soluble proteins and homologous human antisera. The antibodies that specifically recognized the major *L. b. braziliensis* and *L. d. chagasi* surface antigens were eluted from the blot (Fig. 2a, b; lane 1, *arrows*) and incubated with a second blot containing the respective aqueous-phase proteins. The monospecific, eluted antibodies reacted with the major antigens extracted in the aqueous phase (Fig. 2a, b; lane 2) but did not bind to any of the multiple components recognized by the polyvalent sera. These results demonstrate that the membrane and soluble antigens of each parasite species share common antigenic determinants.

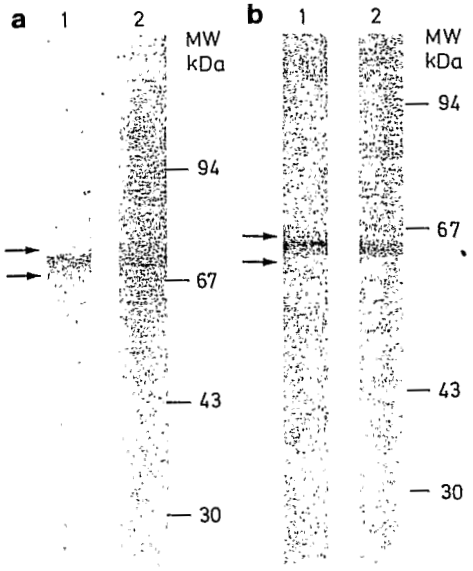

Fig. 2 a, b. Immunological cross-reactivity of the major amphiphilic and hydrophilic antigens. Monospecific antibodies that recognized the major surface-iodinated *L. b. braziliensis* and *L. d. chagasi* antigens present in the respective detergent phases (*lanes 1, arrows*) were eluted from a preparative blot, reacted with a second blot containing the respective aqueous-phase proteins and revealed for peroxidase staining (*lanes 2*). **a** *L. b. braziliensis* reacted with mucocutaneous sera. **b** *L. d. chagasi* reacted with visceral sera

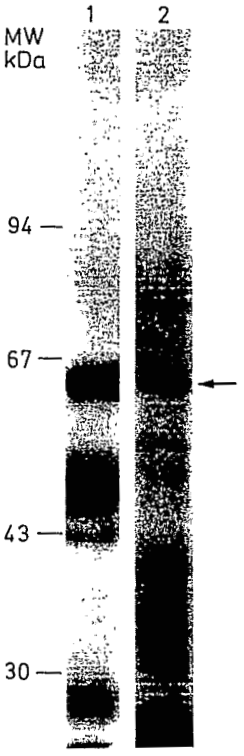

Fig. 3. Autoradiographic patterns from iodinated *L. d. chagasi* amphiphilic and hydrophilic proteins after separation by SDS-PAGE. *Lane 1*, detergent-phase proteins from surface-iodinated *L. d. chagasi* promastigotes. *Lane 2*, aqueous-phase proteins iodinated after Triton X-114 extraction. *Arrows* indicate the position of the major labeled antigens. Gels were exposed for 24 and 12 h, respectively

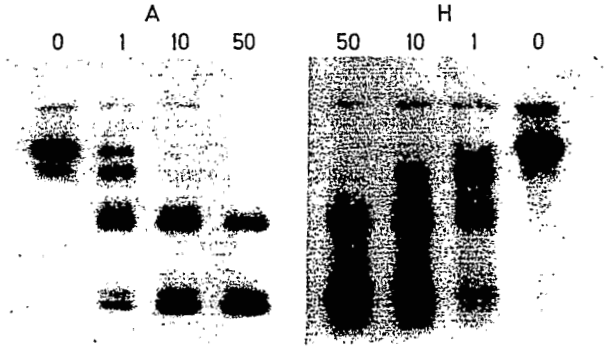

Fig. 4. Peptide mapping of *L. d. chagasi* major amphiphilic and hydrophilic antigens. Labeled 65-kDa amphiphilic (*A*) and hydrophilic (*H*) proteins were excised from the gel shown in Fig. 3 and submitted to proteolytic digestion using different concentrations of *S. aureus* V8 protease (*numbers* indicate the protease concentration in µg/ml). The products of partial proteolysis were separated by SDS-PAGE and autoradiographed

Peptide mapping of membrane and soluble antigens

To determine the possible structural relationship between the amphiphilic and hydrophilic antigens, Cleveland digestion of those proteins was carried out. Surface-iodinated *L. d. chagasi* promastigotes were extracted with Triton X-114 and proteins from the detergent phase were separated. In parallel, non-iodinated parasites were extracted and proteins from the aqueous phase were separately iodinated. The autoradiographic profiles shown in Fig. 3 reveal the labeling of an aqueous-soluble protein with the same molecular weight as the major surface protein of *L. d. chagasi* (Fig. 3). The bands corresponding to the 65-kDa antigens (Fig. 3, *arrows*) were excised from the gels and subsequently processed for proteolytic digestion. The maps generated from the products of partial proteolysis using *S. aureus* V8 protease reveal complete homology in the protein-digestion profiles between the amphibilic antigen and the respective hydrophilic one (Fig. 4). A high degree of homology was also observed by comparing the maps of the 72-kDa amphiphilic and hydrophilic proteins of *L. b. braziliensis* (data not shown). This result indicates that the major membrane and soluble antigens are not only immunologically but also structurally related.

Protease activity of detergent- and aqueous-soluble antigens

The protease activity of proteins extracted with Triton X-114 from surface-iodinated *L. b. braziliensis* and *L. d. chagasi* promastigotes was ana-

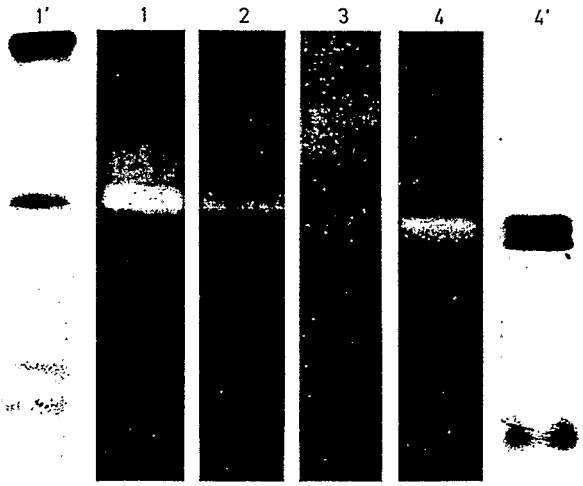

Fig. 5. Proteolytic activity of the amphiphilic and hydrophilic antigens. Detergent- and aqueous-phase proteins from surface-iodinated *L. b. braziliensis* and *L. d. chagasi* promastigotes were separated by SDS-PAGE in a 10% polyacrylamide gel that contained fibrinogen. The gels were stained with Coomassie blue and autoradiographed. *Lanes 1, 2:* proteolytic activity of amphiphilic and hydrophilic *L. b. braziliensis* proteins. *Lanes 4, 3:* proteolytic activity of amphiphilic and hydrophilic *L. d. chagasi* proteins, respectively. *Lanes 1', 4':* the respective autoradiograms of lanes 1 and 4

lyzed by SDS-PAGE in gels containing fibrinogen as the protease substrate (Bouvier et al. 1987). Enzymatic activity against fibrinogen was found in the amphibilic and hydrophilic fractions from both parasite species. In the case of *L. b. braziliensis*, a number of proteolytic activity bands were revealed in the amphiphilic fraction (Fig. 5; lane 1). The higher-activity band was found at the same relative mobility in the hydrophilic fraction (Fig. 5; lane 2) and corresponded to the radioiodinated major surface polypeptide, as shown in the labeling profile of the respective autoradiogram (Fig. 5; lane 1'). In *L. d. chagasi*, two bands of enzymatic activity were identified in the amphiphilic fraction (Fig. 5; lane 4), although they differed in mobility from those in *L. b. braziliensis*. The higher activity found at the same relative mobility in the hydrophilic fraction (Fig. 5; lane 3) also corresponded to the migration of the major radioiodinated protein (Fig. 5; lane 4'). The apparent molecular weight of the major proteases was further assessed by a second electrophoresis after excision of the bands of activity from the gels, rehydration and treatment under denaturating and reducing conditions. The major activities corresponded precisely to iodinated proteins of 72 and 65 kDa from *L. b. braziliensis* and *L. donovani*, respectively (data not shown).

Discussion

The present study demonstrates the presence of hydrophilic proteins, characterized by their partitioning in the aqueous phase of the Triton X-114 extraction system, which are related to the major amphiphilic antigens of *L. b. braziliensis* and *L. d. chagasi*. These hydrophilic proteins were first identified by immunoblotting with homologous human antisera, which recognized 72- and 65-kDa antigens in both detergent and aqueous phases. For both parasite species, the identity of the membrane and soluble major antigens was confirmed by their cross-reactivity, inasmuch as eluted antibodies that specifically recognized the membrane form of the protein also recognized the soluble protein, and by the homology found in their peptide-digestion profiles.

Bouvier and co-workers (1985) previously identified a soluble form (present in small amounts) of the major amphiphilic surface antigen of *L. major* appearing during its purification. Since the purification in that study started from extracted proteins occurring in the detergent phase, the authors concluded that the soluble form was generated during the purification of the amphiphilic protein by the hydrolysis of its hydrophobic membrane anchor. However, the hydrophilic proteins identified in the present study do not seem to be hydrolytic products of the amphiphilic antigen, but rather proteins normally occurring in the promastigote cytosol, for the following reasons: (a) the proteins present in both phases were analyzed directly after the extraction of promastigotes; (b) starting with surface-iodinated cells, no radioactive proteins were found in the aqueous phase, thus ruling out the possibility of either an incomplete phase separation or a hydrolytic product generated during the lysis and extraction of the cells; (c) the presence of the antigens in the aqueous phase of the extract is quantitatively significant as revealed by densitometric analysis of the immunoblots and of gels stained with Coomassie blue.

The amphiphilic and hydrophilic 72- and 65-kDa antigens in *L. b. braziliensis* and *L. d. chagasi*, respectively, are also functionally related inasmuch as both display proteolytic activity. The latter has previously been demonstrated by Etges et al. (1986) in *L. major* for the purified membrane form of the antigen as well as for the soluble form identified after phospholipase C treatment of the membrane antigen. The presence of soluble proteases was formerly reported by Coombs (1982), who found proteolytic activity in particulate and soluble fractions of promastigotes and amastigotes

of *L. m. mexicana*. Pupkis and Coombs (1984) have suggested that a soluble 67-kDa protease may be common to both forms of the parasite.

In contrast to our findings, Bouvier et al. (1987) could not find any correlation between proteolytic activity and the major surface-iodinated protein of *L. b. braziliensis*. This divergence from the present results can be attributed to differences in the strains used. At a lower pH of 4–4.5, which was the optimal pH reported for the *L. m. amazonensis* enzyme (Chaudhuri and Chang 1988), proteolytic activity could not be observed in the detergent phase of both parasites, and only low-molecular-weight components in the aqueous phase displayed proteolytic activity (data not shown).

Based on the results presented in this communication, we can conclude that the membrane and soluble major antigens present in *L. b. braziliensis* and *L. d. chagasi* promastigotes are closely related. The hydrophilic antigen could have originated by several mechanisms, such as an endogenous phospholipase-mediated cleavage of the phospholipid that anchors the promastigote amphiphilic surface protein. However, our data and the fact that no such phospholipase activity has yet been found in *Leishmania* exclude this possibility. Another possible mechanism could be a post-translational modification that enables the sorting-out of the antigen to its final destination in the cell. In that case, the proteins are synthesized in their soluble form and only a number of them are processed by the attachment of a phospholipid anchor upon their insertion into the plasma membrane. This assumption can be related to the findings that the major surface protease of promastigotes is not present on the amastigote surface (Fong and Chang 1982; Chang et al. 1986; Heath et al. 1987), although it has been found to be immunogenic during animal and human infection with *Leishmania* (Lepay et al. 1983; Legrand et al. 1987; Etges et al. 1985; Colomer-Gould et al. 1985). Moreover, Heath et al. (1987) recently demonstrated by in vitro translation that *L. donovani* promastigotes and amastigotes possess the mRNA that directs the synthesis of a 63-kDa protein, which is recognized by human kala-azar sera in both stages of parasite development.

It has been proposed that the promastigote major surface antigen is a key surface molecule involved in parasite-macrophage interaction and in the initial phases of the infection (Chang and Chang 1986). The soluble form of the antigen may eventually be secreted, thereby increasing the proteolytic effect of its membrane congener. This could facilitate the degradation of macrophage surface and/or cytoplasmic proteins, thus evading the immunological mechanisms required for parasite destruction inside the macrophage.

Last but not least, these cytoplasmic antigens, which are demonstrably abundant in promastigotes, could serve as an adequate source for further purification towards their future use in the diagnosis and therapy of leishmaniasis.

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