SURFACE ANTIGENS OF *LEISHMANIA DONOVANI* PROMASTIGOTES*

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Leishmania donovani, the agent of visceral leishmaniasis, is a trypanosomatid protozoan transmitted by phlebotomine sandflies. The parasite cycles between a nonmotile, intracellular amastigote stage parasitizing the mononuclear phagocytes of the mammalian host and an extracellular, motile promastigote stage in the insect vector. A similar promastigote form develops when parasites are cultured in cell-free medium at 26–28°C (1) and remains infective when injected into susceptible hosts (2).

In humans, *L. donovani* produces a range of disease states (3). The classic kala-azar of India is a disease of adults with no known animal reservoir and association with post-kala-azar dermal lesions. Sudanese kala-azar also affects adults, but shows little tendency toward dermal sequelae, some heightened resistance to antimony treatment, and has its natural reservoir in the rat. Lastly, the Mediterranean and South American variants are diseases of childhood with dogs as frequent reservoirs. Efforts to correlate these clinical and epidemiological manifestations with specific *Leishmania donovani* strains and isolates have led to numerous classification schemes. These depend upon comparisons of DNA buoyant density (4), electrophoretic patterns of isoenzymes (5, 6), serologic methods directed at excreted factors (7), and examination of the surface proteins of cultured parasites (8). To date, none has been successful in identifying antigens common to all or a clinically significant subset of *L. donovani* isolates.

In this paper, we report that the surface antigen profiles of *Leishmania donovani* promastigote isolates from Brazil and Africa are remarkably similar and extremely simple. A glycoprotein of 65,000 mol wt is the principal component identified by both surface iodination and biosynthetic labeling. Recognition of this 65,000 mol wt glycopeptide by human immune sera from diverse geographic locations establishes it as a major immunogenic determinant common in at least two clinical variants of leishmaniasis.

Materials and Methods

Parasites. Khartoum and Sudan 1S strains of *Leishmania donovani* were generously provided by Dr. Jan Keithly (Cornell University Medical College, New York). The Brazilian strains represent local isolates of kala-azar from a human case from Bangu, Rio de Janeiro (HBI) and a canine case from Campo Grande, Rio de Janeiro (CGD) and were obtained from Dr. Mauro Marzochi (Fundação Oswaldo Cruz, Rio de Janeiro, Brazil) (9). Parasites were maintained by serial passage in Syrian golden hamsters and promastigotes obtained by culture of splenic homogenates in Schneider's Drosophila medium (Gibco Laboratories, Grand Island, NY) supplemented with 30% fetal calf serum (Hyclone; Sterile Systems Inc., Logan, UT) (heat-

inactivated for 30 min, 56°C), 100 U/ml penicillin G, and 100 μg/ml streptomycin at 26°C (10). An initial centrifugation (100 g for 10 min at 4°C) was performed to clear homogenates of erythrocytes and tissue debris before culturing. For surface iodination and biosynthetic labeling, promastigotes were passaged at 5-d intervals in supplemented Schneider's Drosophila medium (as above) and used on the 3rd d of the second to fifth passage. Infectivity was established by periodic reinoculation of 5 × 10^7 cultured promastigotes into Syrian hamster hosts.

Cell Surface Iodination. Promastigotes were washed three times by centrifugation (500 g for 10 min at 4°C) in Ca^{++} and Mg^{++}-free phosphate-buffered saline (PD) and surface proteins radioiodinated using a modification of the lactoperoxidase-glucose oxidase procedure of Hubbard and Cohn (11). Lactoperoxidase was obtained from Calbiochem-Behring Corp., American Hoechst Corp., La Jolla, CA; glucose oxidase (Type V) was procured from Sigma Chemical Co., St. Louis, MO; and carrier-free Na (^{125}I) was purchased from New England Nuclear, Boston, MA. After radioiodination, >95% of the promastigotes remained viable and actively motile. Parasites were washed free of radiolabel and solubilized in lysis buffer (phosphate-buffered saline [PBS][1] containing 0.5% Nonidet P-40 [Bethesda Research Laboratories, Bethesda, MD], 100 U/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride [Sigma Chemical Co.]).

Biosynthetic Labeling with [^{35}S]Methionine. 2 × 10^8 promastigotes were washed twice by centrifugation (500 g for 10 min at 4°C) in serum-free Schneider's Drosophila medium followed by a single wash in methionine-free Schneider's medium (Gibco Laboratories). Parasites were then incubated at 26°C for 12 h in 5 ml of methionine-free Schneider's medium containing 1 mg/ml bovine serum albumin (BSA) and 100 μCi of [^{35}S]methionine (500 Ci/mmol) (Amersham Corp., Arlington Heights, IL). After washing free of unincorporated radiolabel, aliquots of live ^{35}S-labeled parasites were removed for immune precipitation of surface components. The remaining promastigotes were solubilized in lysis buffer (as above) and immunoprecipitated as whole lysates.

Immune Precipitation. ^{125}I- and ^{35}S-labeled promastigote lysates were centrifuged to remove nuclei and debris (23,000 g for 20 min, 4°C) and 50-μl aliquots of the clarified supernatant were incubated with 5 μl of human immune serum (1 h at 4°C with constant agitation). Immune complexes were isolated as described previously (12, 13). Antigens were eluted in 50 μl of electrophoresis sample buffer (2% sodium dodecyl sulfate [SDS], 12% sucrose, 0.01% bromophenol blue, 50 mM dithiothreitol, and 50 mM Na_2CO_3 buffer, pH 8.6) and boiled for 2 min. Samples were stored at −70°C until used.

For isolation of [^{35}S]methionine-labeled surface components, live, radiolabeled promastigotes were resuspended in 0.5 ml of PBS containing 100 μl of human immune serum and incubated for 30 min at 4°C with gentle agitation. After washing by centrifugation (500 g for 10 min), lysates were prepared, clarified by centrifugation (23,000 g for 20 min), and immune complexes were isolated as above.

Lectin Binding. 50-μl aliquots of labeled lysates were incubated with 50 μl of a 30% vol/vol suspension of concanavalin A (Con A)-Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, NJ) for 1 h at 4°C with constant agitation. Samples were centrifuged, washed twice in salt-azide buffer at 4°C, twice in detergent solution at room temperature (0.05% NP-40, 0.3 M NaCl, 10 mM Tris-HCl, pH 8.6), and a final time in salt azide buffer. Samples were eluted in 40 μl of electrophoresis sample buffer and boiled for 2 min. Nonspecific binding was assessed by incubating samples in the presence of 0.25 M α-methyl mannoside (Sigma Chemical Co.).

Polyacrylamide Gel Electrophoresis. Radiolabeled proteins were electrophoresed in 1-mm-thick, 4–11% polyacrylamide gradient slab gels (14). Gels of ^{35}S-labeled proteins were processed for fluorography (15) and exposed on prefogged Kodak X-Omat R-1 film (Eastman Kodak Co., Rochester, NY) at −70°C (16). ^{125}I gels were autoradiographed using Kodak X-Omat R-1 film with Dupont Kronex Lightning Plus intensification screens (DuPont Instruments, Wilmington, DE).

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; CDG, canine strain of *L. donovani*; Con A, concanavalin A; HBI, human Bangu isolate strain of *L. donovani*; NHS, nonimmune human serum; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

Human Immune Serum.   Human immune serum from eight patients hospitalized with kala-azar (K1-4) or cutaneous disease (*L. braziliensis*, T1-4) were generously provided by Dr. Mauro Marzochi and Dr. Keyla Marzochi (Dept. of Tropical Medicine, Hospital das Clinicas, Federal University of Rio de Janeiro, Brazil). Patients were recruited from a local focus of infection (17), and clinical histories are available. *L. donovani* immune serum was additionally obtained from six patients in East Africa (A1-6), diagnosed as kala-azar but without detailed clinical documentation. Sera were heat inactivated (56°C for 30 min) before incubation with live biosynthetically labeled promastigotes.

Sera for Cross-Reactivity Studies.   Rabbit anti-BSA was purchased from Cappel Laboratories (Cochranville, PA). *Trypanosoma cruzi* immune IgG was purified by sodium sulfate precipitation of plasma from patients hospitalized with chronic Chagas' cardiopathy. This was made possible through the cooperation of Dr. Sonia Andrade and Dr. Zilton Andrade (Federal University of Bahia, Brazil). Syphilis (*Treponema pallidum*) immune sera were provided by Dr. Alberto Agosta (Cornell University Medical College, New York) and confirmed to be both FTA-ABS- and VDRL-positive. Leprosy (*Mycobacterium leprae*) immune sera were obtained from Dr. Mark Kaplan (North Shore University Hospital, Manhasset, NY).

Results

Externally Disposed Polypeptides of L. donovani Promastigotes—Brazilian Strains

HUMAN BANGU ISOLATE (HBI).

Surface iodination profile.   Promastigotes of *L. donovani* obtained through culture of a HBI from Brazil were surface iodinated with labeled polypeptides subjected to 4–11% gradient SDS-polyacrylamide gel electrophoresis (PAGE). The resulting pattern, shown in Fig. 1, lanes 1 and 2 illustrates the presence of relatively few surface polypeptide components. The major peptide displays an apparent molecular weight of 65,000 and occurs in association with a less distinct band of 59,000 mol wt. Consistent also is a doublet occurring at 23,000 and 25,000 mol wt. A weakly labeling band seen sporadically at 50,000 mol wt is discussed below.

Recognition by human sera.   Immunoprecipitation patterns for HBI strain promastigotes using immune sera from four Brazilian patients with kala-azar (K1-4) are illustrated in Fig. 1, lanes 4–7. The patterns are clearly consistent and demonstrate that the 65,000-mol wt peptide is strongly recognized by all immune sera. Bands at 23,000, 25,000, and 59,000 mol wt are markedly reduced in intensity and only identified with prolonged autoradiograph exposures (note the difference in exposure time between the right and left halves of Fig. 1).

Particularly striking is the marked similarity of the K1-4 immunoprecipitation patterns to those obtained from patients with cutaneous disease (T1-4; Fig. 1, lanes 8–11). Clearly, the 65,000 mol wt peptide is a major immunogenic determinant recognized by tegumental as well as kala-azar immune sera. Incubation of lysates with normal, nonimmune human serum (NHS) does not precipitate any labeled bands (Fig. 1, lane 3).

CANINE STRAIN (CGD).

Surface iodination pattern.   Promastigotes from a second Brazilian isolate of *L. donovani*, this from a dog (CGD), were similarly examined for externally disposed polypeptides. The iodination profile, displayed in Fig. 2, lanes 1 and 2, shows the major surface peptide at 65,000 mol wt, a doublet at 23,000 and 25,000 mol wt, and a number of minor bands. The seeming identity of patterns from HBI and CGD strains is apparent when lysates are electrophoresed in parallel (data not shown).

Recognition by human sera.   Immunoprecipitation of CGD strain promastigotes with
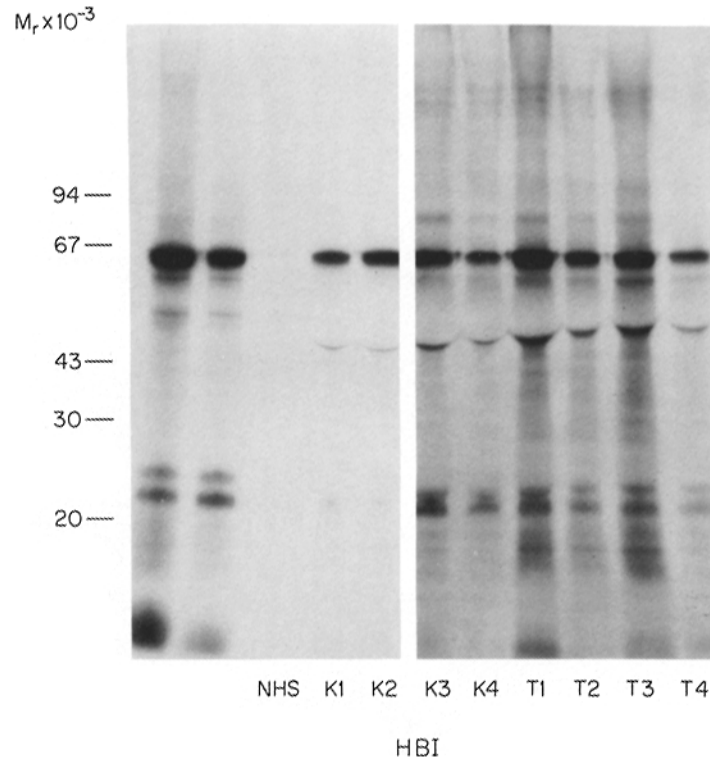
$M_r \times 10^{-3}$

HBI

Fig. 1. Surface iodination and immunoprecipitation profiles of Brazilian HBI strain promastigotes. Lanes 1 and 2: surface iodination profile; lane 3: precipitation of ^{125}I-labeled whole cell lysate with NHS; lanes 4–7: ^{125}I-labeled whole cell lysates with kala-azar immune sera from Brazilian patients (K1-4); lanes 8–11: ^{125}I-labeled whole cell lysates with immune sera from cutaneous leishmaniasis patients (T1-4; *L. braziliensis*). Autoradiograph exposure times were 24 h (left) and 96 h (right). M_r, molecular weight.

kala-azar immune sera from Brazil (Fig. 2, lane 10) reveals a single major peptide analogous to that recognized on HBI promastigotes. Using a battery of sera from East African patients diagnosed with visceral leishmaniasis (A1-6; Fig. 2, lanes 4–9), the 65,000 mol wt band is strongly recognized in five of six cases. A similar result is seen when HBI strain promastigotes are immunoprecipitated with East African immune sera (not shown). The lack of specific clinical information on patient A1 highlights the question of diagnosis in explaining the absence of reactivity.

Externally Disposed Polypeptides of L. donovani Promastigotes—African Strains.

SUDAN AND KHARTOUM STRAINS.

Surface iodination profile. To assess the possibility of strain variations between parasites from diverse geographic locations, the labeling pattern of Sudan 1S strain promastigotes was examined. Fig. 3, lanes 1 and 2 illustrate the remarkable preservation of profiles seen between lysates from African and Brazilian strains. Present are the 65,000-mol wt polypeptide along with the doublet at 23,000 and 25,000 mol wt and several minor bands. A second African strain from Khartoum, while radiolabeling only weakly, displays a similar pattern with increased prominence of the minor bands at 59,000 and 85,000 mol wt (not shown).
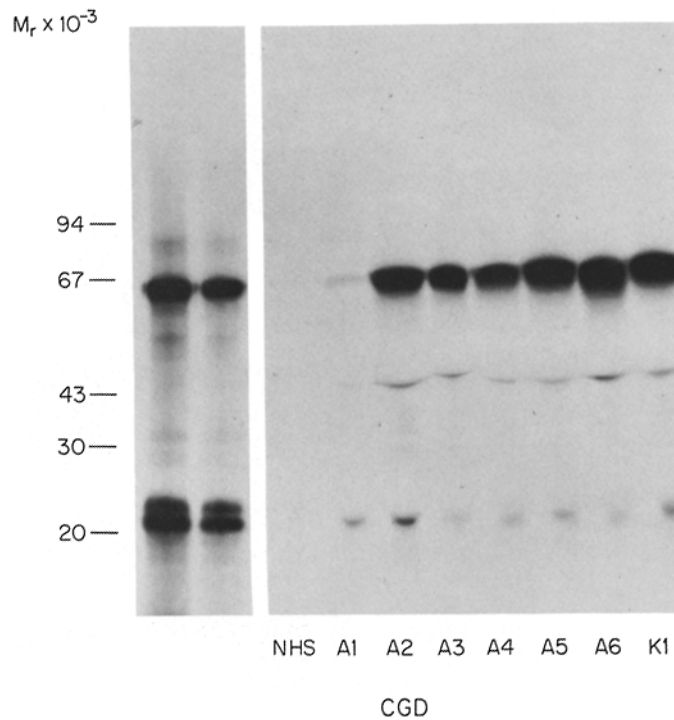
$M_r \times 10^{-3}$

NHS A1 A2 A3 A4 A5 A6 K1

CGD

FIG. 2. Surface iodination and immunoprecipitation profiles of Brazilian CGD strain promasti-gotes. Lanes 1 and 2: surface iodination profile; lane 3: ^{125}I-labeled whole cell lysate precipitated with NHS; lanes 4–9: iodinated whole cell lysates immunoprecipitated with kala-azar immune sera from East African patients (A1-6); lane 10: iodinated lysates with kala-azar immune serum from Brazilian patient K1. M_r, molecular weight.

Recognition by Human Sera. Analogous to the whole lysate patterns, immunoprecipitation profiles show a seeming identity between African and Brazilian strains. In all but one case, immune sera from both areas bring down a single major peptide of 65,000 mol wt from Sudan 1S lysates (Fig. 3, lanes 3–9). This pattern is further repeated in immunoprecipitates using Khartoum strain promastigotes.

Biosynthetic Labeling of L. donovani Promastigotes.

To establish the parasitic origin of the iodinated polypeptides and to examine for peptides unavailable for surface iodination, biosynthetic labeling was performed. After incubation of promastigotes for 12 h in the presence of [^{35}S]methionine, sufficient label was incorporated to yield the peptide profiles illustrated for HB1 and Khartoum strains (Fig. 4, A lanes). Immunoprecipitation of these whole lysates with serum from patient K1 highlights only a small number of these bands (Fig. 4, C lanes). In particular, a band at 65,000 mol wt stands out sharply in both African and Brazilian strains. Also prominent is a peptide of 85,000 mol wt corresponding to the weakly iodinated band recognized in Fig. 1. Few peptides are recognized by NHS (Fig. 4, B lanes), and these only weakly.

While Fig. 4 illustrates immunoprecipitation patterns using whole lysates of biosyn-thetically labeled parasites, surface components were additionally identified by incu-bating viable ^{35}S-labeled promastigotes with kala-azar immune sera. After incubation
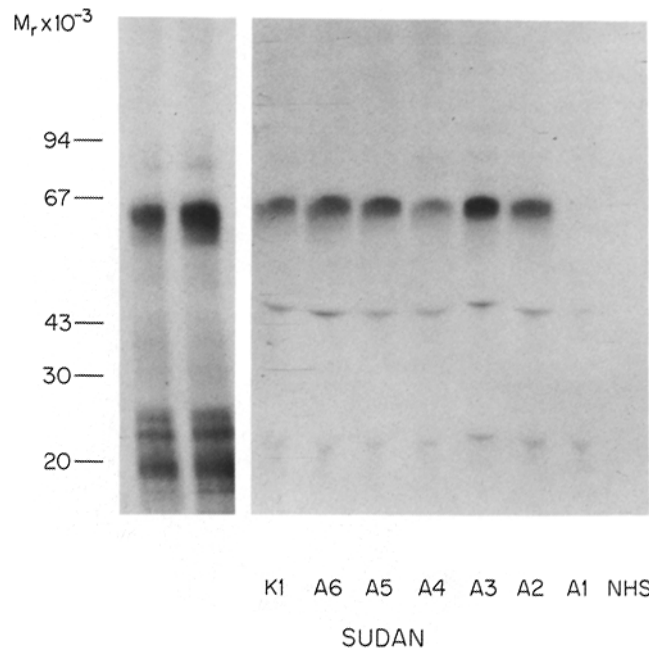
FIG. 3. Sudan 1S strain promastigotes: surface iodination and immunoprecipitation patterns. Lanes 1 and 2: surface iodination profile; lane 3: iodinated promastigote lysate immunoprecipitated with Brazilian kala-azar immune serum K1; lanes 4–9: iodinated lysates immunoprecipitated with East African kala-azar immune sera (A1-6); lanes 10: iodinated lysate with NHS. M_r, molecular weight.

at 4°C for 30 min, parasites were washed, lysed, and immune complexes were isolated. In these experiments, faint bands at 65,000 and 85,000 mol wt were clearly and uniquely present, establishing the external disposition of these peptides (data not shown).

Binding to Con A-Sepharose 4B.

Some evidence as to the glycopeptide nature of the 65,000-mol wt component was obtained by incubating iodinated lysates with Con A-Sepharose 4B. Examination by SDS-PAGE (Fig. 5, Con A; illustrated for Sudan 1S strain promastigotes) reveals strong binding of both 65,000-mol wt and 23,000/25,000-mol wt doublet to Con A-Sepharose. Such binding is specifically although incompletely blocked by addition of 0.25 M α-methyl mannoside (Fig. 5, α-MM).

Cross-Reactivity with Other Sera.

The proximity of our 65,000-mol wt band to BSA led us to investigate the separate identity of the two. Using a rabbit anti-BSA reagent, a faint but unique band was precipitated (67,500 mol wt; not shown). In a similar manner, the specificity of the 65,000 mol wt peptide for *L. donovani* was further confirmed using a battery of immune sera from leprosy, syphilis, and Chagas' disease patients. In only one instance (a Chagas' patient) was cross-reactivity faintly detected, and this only with prolonged autoradiograph exposure (data not shown).

Discussion

Our aim in this study has been to examine the surface antigens of *Leishmania donovani* promastigotes recognized by human immune sera as a function of geographic
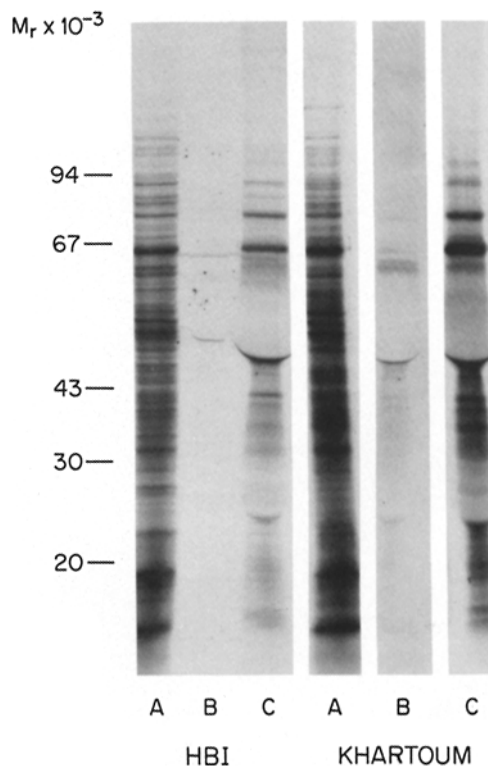
FIG. 4. Biosynthetic labeling of HBI (Brazilian) and Khartoum strain promastigotes with [^{35}S]methionine. A lanes: whole cell lysates, 12 h incorporation; B lanes: whole cell lysates precipitated with NHS; C lanes: whole cell lysates immunoprecipitated with kala-azar immune serum from Brazilian patient K1. M$_r$, molecular weight.

diversity. Using surface radioiodination, we have demonstrated a similar, uncomplicated peptide profile for each of four promastigote strains, two from Brazil and two from Africa. In each case, precipitation of lysates with kala-azar immune serum brings down a major immunogenic determinant of 65,000 mol wt. Immune sera from African and Brazilian patients are equally effective in recognizing this peptide, suggesting no major differences in surface make-up among geographically distinct strains.

Biosynthetic labeling with [^{35}S]methionine reveals a predictably greater number of labeled proteins. However, immunoprecipitation of live promastigotes and promastigote lysates highlights the same 65,000-mol wt surface peptide in both African and Brazilian strains. That this antigen may be a glycopeptide is suggested by specific binding of iodinated lysates to Con A.

The consistency of these surface polypeptide patterns is maintained not only between strains but also with time in culture. Indeed, no change in whole lysate or immunoprecipitation profile has been noted with cultures up to 8 d of age and with serial passage of promastigotes over the course of 3 mo. Infectivity is likewise undiminished with culture age, confirming an earlier observation by Keithly and Bienin (18).

Using immune sera from four patients with cutaneous leishmaniasis, the 65,000-mol wt antigen is similarly recognized on both African and Brazilian isolates of *L.*
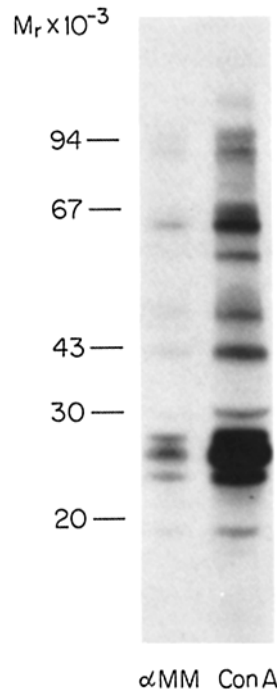
$M_r \times 10^{-3}$

αMM ConA

Fig. 5. Binding of iodinated surface peptides of Sudan 1S strain promastigotes to Con A-Sepharose 4B (Con A lane). α-MM lane: specific inhibition of Con A binding by addition of 0.25 M α-methyl mannoside. M_r, molecular weight.

donovani. In this regard, Hernandez et al. (19) have reported a virtually identical surface iodination profile for promastigotes of *Leishmania braziliensis*, an agent of cutaneous leishmaniasis. Here a major glycoprotein of 65,300 mol wt is seen together with a band of 54,000 mol wt and a doublet of 23,000/25,000 mol wt. Similarly, Chang and Fong (20) have identified a glycopeptide triplet of 65,000–68,000 mol wt recognized by monoclonal reagents on promastigotes of *Leishmania mexicana amazonensis.* Moreover, Handman et al. (21) have reported a promastigote-specific surface peptide of 50,000 mol wt under nonreducing conditions in isolates of *L. tropica.* In our own experiments with nonreducing conditions, we have noted a shift in the major immunogenic determinant from 65,000 to 50,000 mol wt (data not shown). A faint but sporadic band at 50,000 mol wt in our reduced gels may similarly represent incomplete reduction of sulfhydryl groups.

The possibility of a common major surface antigen not only between *Leishmania donovani* strains but between *Leishmania* species in general is presently being explored by detailed peptide mapping. Indeed, the association of surface glycoproteins with enzymatic activities as demonstrated for acid phosphatase on *L. donovani* (22) and neuraminidase on *T. cruzi* (M. Pereira and N. Nogueira, manuscript in preparation) suggests at least one mechanism whereby parasites with a common surface profile could produce multiple disease states. Conceivably, these surface glycopeptides could represent families of isoenzymes, sharing immunogenic determinants among members but with differing substrate specificities or specific activities accounting for strain-specific behavior. In addition, small differences in glycosylation could well account

for differences in immune recognition between *Leishmania* species. Alternatively, host differences, either genetic or environmental, could be responsible for the particular clinical presentation.

From the standpoint of diagnostics, the most commonly used serologic tests for kala-azar utilize indirect immunofluorescence (23, 24) or an enzyme-linked immunosorbent assay technique (24, 25). Both show high sensitivity but lack specificity, exhibiting cross-reactivity for trypanosomes and mycobacteria. In this regard, the virtual lack of recognition of the 65,000 mol wt peptide by immune sera from leprosy, syphilis, and Chagas' disease patients may be significant. With existing technology for monoclonal antibody production, a more specific serologic procedure directed at the 65,000-mol wt surface polypeptide may be feasible.

Likewise, the use of *Leishmania* antigens in vaccine development is an area of ongoing interest. As the infective stage, the promastigote seems an obvious target in immunoprophylaxis. Indeed, Howard et al. (26) have recently demonstrated significant protective immunity in genetically susceptible mice to *L. tropica* infection by prophylactic immunization with irradiated promastigotes. Clearly, an assessment of protective immunity induced by the 65,000 mol wt peptide as well as the possibility of large scale isolation of this component through recombinant DNA technology is of importance and is currently under investigation.

Summary

Surface antigen profiles of *Leishmania donovani* promastigote isolates have been studied. Surface patterns of Brazilian and African isolates display remarkable similarities and are extremely simple, consisting of three major peptides of 65,000, 25,000, and 23,000 mol wt.

Surface iodination and biosynthetic labeling coupled to immunoprecipitation techniques revealed that a single major determinant of 65,000 mol wt is recognized in all strains by sera from kala-azar patients from both Brazil and Africa. This major determinant is not brought down by sera from normal individuals and shows no significant cross-reactivity with sera from Chagas' disease, leprosy, or syphilis patients. Binding to concanavalin A suggests a glycoprotein nature for this antigen.

Sera from patients with cutaneous leishmaniasis (*L. braziliensis*) also recognized the same 65,000-mol wt determinant, although to a lesser extent. The possibility that this major surface antigen is shared, with minor differences, not only by *L. donovani* strains but between *Leishmania* species in general is suggested.

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