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Characterization of Integral Membrane Proteins of *Leishmania major* by Triton X-114 Fractionation and Analysis of Vaccination Effects in Mice

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The total integral membrane proteins of promastigotes of *Leishmania major* were extracted by using the Triton X-114 phase separation technique and were characterized by immunoprecipitation, Western blotting (immunoblotting), and lectin chromatography. Of the 40 or more proteins which partitioned into the detergent phase, only about 10 proteins could be surface radioiodinated on live promastigotes, suggesting their surface orientation. The abundance of the gp58-63 antigen varied markedly between two strains of *L. major*. Sera from patients with visceral leishmaniasis caused by *Leishmania donovani chagasi* recognized the gp58-63 complex and an additional M_r-42,000 polypeptide shared between *L. major* and *L. donovani chagasi*. A subpopulation of six surface proteins, including the abundant gp58-63 antigen and a group of proteins of M_r 81,000 to 105,000, were glycoproteins recognized by antiserum to wheat germ agglutinin- or concanavalin A-binding proteins. The membrane proteins of the LRC-L119 isolate of *L. major* could successfully vaccinate genetically susceptible mice, thus opening the way for a molecularly defined subunit vaccine composed of glycolipid and membrane protein antigens.

Leishmania major, the etiological agent of Old World cutaneous leishmaniasis, is transmitted to vertebrates as the flagellated, promastigote stage by phlebotomine sandflies (8). Promastigotes parasitize host macrophages by a receptor-mediated mechanism and convert to the nonflagellated, amastigote stage within the phagolysosome (8).

Promastigote cell surface molecules are critical for recognition and infection of the mammalian host and possibly for subsequent survival in the vector. To date, two surface molecules from the *L. major* promastigote have been analyzed in detail. The lipophosphoglycan (LPG) of *L. major* has been shown to be involved in parasite attachment to macrophages (17, 27, 31). This molecule has also been shown to successfully vaccinate mice against infection with *L. major* (19, 27). A second, well-characterized membrane antigen is the major *Leishmania* surface glycoprotein gp63. This is an M_r-63,000 to -65,000 protease, anchored to the parasite membrane with a phosphatidyl inositol glycolipid anchor (3, 11, 14).

Since parasite membrane antigens are the interface between the parasite and its vertebrate and insect hosts, we set out to identify and characterize the promastigote membrane proteins of *L. major* by using the Triton X-114 (TX-114) detergent phase separation technique of Bordier (2) combined with lectin chromatography. This study has shown that the pattern of promastigote membrane glycoproteins is relatively simple and that this fraction of the total cell proteins can vaccinate and protect genetically susceptible mice from lethal infection with *L. major*.

MATERIALS AND METHODS

Parasites. The virulent cloned line *L. major* LRC-L137/7/V121 (MHOM/1L/67/Jericho II) (termed V121) was isolated as previously described (18). An avirulent cloned line *L. major* LRC-L119.E4.B2 derived from the uncloned strain *L. major* LRC-L119 isolated in Kenya was used for some of the studies described (21). Parasites were maintained by passage in vivo in BALB/c mice or as promastigotes in vitro in Schneider's drosophila medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum.

TX-114 preparation of integral membrane proteins. Cell lysates were prepared by a modification of the detergent phase separation method of Smythe et al. (34), which is a modification of the original method of Bordier (2). Stationary-phase promastigotes (10^{10}) were solubilized in 80 ml of 0.5% TX-114 in phosphate-buffered saline (PBS; Fluka Chemie AG, Buchs, Switzerland), pH 7.3, homogenized in a Dounce homogenizer to break up the parasites, and kept on ice for 90 min. A cocktail of protease inhibitors was added to the parasite lysate during lysis consisting of 1 μg each of phenylmethylsulphonyl fluoride, pepstatin, chymostatin, antipain, iodoacetamide, and leupeptin (Sigma Chemical Co., St. Louis, Mo.) per ml. Insoluble material was removed from the lysate by centrifugation at 37,000 × *g* for 35 min in a Sorvall SS34 rotor at 4°C. The remainder of the procedure is as described by Smythe et al. (34).

Preparation of antisera to lectin-binding material. Promastigote lysates were prepared as described above in a lectin buffer containing 0.9% NaCl, 10 mM Tris hydrochloride, pH 7.3, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 1% TX-114. Lysates were passed twice over a 2-ml column of concanavalin A (ConA)-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) preequilibrated in lectin buffer in order to deplete the lysate of proteins containing mannose and glucose, which bind ConA. The material which did not bind ConA any longer was passed over a 2-ml wheat germ agglutinin (WGA)-Sepharose column to enrich for proteins containing *N*-acetylglucosamine, which bind WGA (Pharmacia). After the column was washed with 10 column volumes of lectin buffer, bound material was eluted from the ConA column by using 0.1 M α-methyl-mannoside and from the WGA column by using *N*-acetylglucosamine. Both buffers contained 1% TX-114 in 0.02 M sodium phosphate, pH

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7.3–0.148 M NaCl (PBS). The protein content was estimated by using the Pierce protein detection kit (Pierce Chemical Co., Rockford, Ill.). Rabbits were injected subcutaneously with 100 μg of ConA-binding proteins or WGA-binding proteins emulsified in Freund complete adjuvant. Several weeks later, the animals were given booster injections of protein alone, and they were bled 1 week later.

Immunoblotting of parasite material. Samples were added to sodium dodecyl sulfate (SDS) sample buffer (0.0625 M Tris hydrochloride [pH 6.8], 50 mM dithiothreitol, 10% glycerol, 0.07 M SDS) and boiled for 5 min, separated by polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels (25), and blotted electrophoretically onto nitrocellulose membranes (0.22-μm pore size; Schleicher & Schuell, Dussel, Federal Republic of Germany) as described by Burnette (6). The membranes were incubated in 5% skim milk in PBS (BLOTTO; 23) to block available binding sites and then incubated with antibodies as described in the text. Radioiodinated protein A (specific activity, 40 μCi/μg) was used to detect immune complexes. For maximum detection of TX-114 detergent phase antigens on Western blots, methanol precipitation was necessary to remove the TX-114 which caused anomalies in electrophoresis and protein transfer and to concentrate the small amounts of protein present in this phase. For precipitation, approximately 9 volumes of ice-cold methanol (−20°C) was added to the detergent droplet resulting from TX-114 phase separation and left overnight at −20°C. Protein was recovered by centrifugation at 10,000 rpm for 30 min at −10°C in a Sorvall HB-4 rotor. The protein pellet was drained and suspended directly in SDS sample buffer prior to SDS-PAGE.

Radioiodination of *L. major* promastigotes. *L. major* promastigotes were radiolabeled by using lactoperoxidase-catalyzed iodination as previously described (20).

Immunoprecipitations. Parasite lysates were immunoprecipitated as described by Handman et al. (20). Briefly, the detergent phase following TX-114 phase separation was reconstituted to 0.5% TX-114 in PBS and incubated with washed *Staphylococcus aureus* Cowan 1 (24) to remove molecules binding *S. aureus* alone. Precleared lysates were incubated with various sera at a 1:10 ratio on ice for 1 h. Immune complexes were collected by using *S. aureus* as described previously (24).

Peptide mapping by limited proteolysis. Peptide mapping was performed as previously described (20) by using staphylococcal V8 protease in a modification of the method of Cleveland et al. (9). Briefly, after the autoradiography of the immunoprecipitates described in Fig. 3, the M_r-58,000 band was cut out from the dry gel and placed in the stacking gel of a new 15% acrylamide gel. The well containing the gel piece was filled with 20 μg of V8 protease (Sigma) per ml in SDS sample buffer (25), and digestion was performed for 15 min at room temperature. Electrophoresis was allowed to proceed until the bromophenol blue dye reached the main gel; the power was then turned off, and proteolytic digestion was continued for an additional 30 min. When electrophoresis was complete, the gel was dried and autoradiographed at −70°C with Cronex Lightning-Plus (Du Pont Co., Wilmington, Del.) intensifying screens and Agfa Curix RP-2 film.

Vaccination studies. BALB/c *H-2*^{k} mice were used for the vaccination experiments. Mice of this genotype are intermediate in their susceptibility to disease caused by *L. major* (29). Groups of 16 experimental and 8 control mice were injected intraperitoneally twice with approximately 200 μg of TX-114 phase membrane proteins purified from the avirulent strain *L. major* LRC-L119 as described in the text, together with 200 μg of the adjuvant *Corynebacterium parvum* (Wellcome Research Laboratories, Beckenham, England [Div. Burroughs Wellcome Co.]) or in Freund complete adjuvant (GIBCO). Injections were given at 4-week intervals. Two weeks after the last injection, mice were infected intradermally with 10^6 virulent promastigotes of *L. major* V121, and lesion development was assessed weekly (29) and expressed as mean lesion score plus or minus the standard error of the mean. As a control for the vaccinating ability of the proteins, one group of mice was injected after treatment of the antigen for 1 h at 37°C with 100 μg of pronase (Calbiochem-Behring, La Jolla, Calif.) per ml. The booster was treated with 100 μg of proteinase K per ml for 1 h at 37°C.

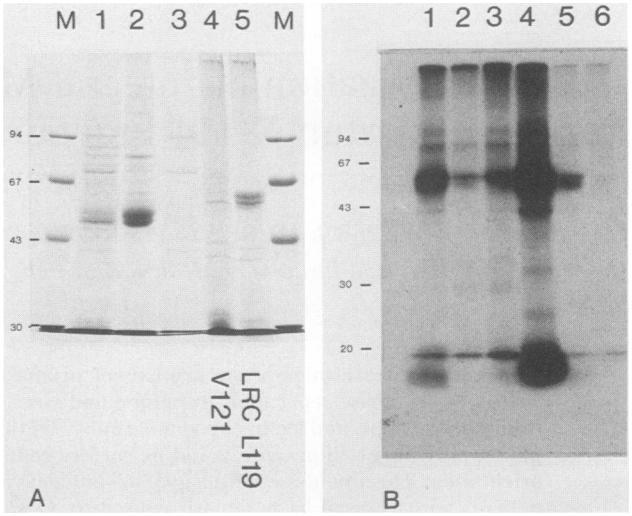

FIG. 1. TX-114 fractionation of proteins from promastigotes of *L. major*. (A) Coomassie brilliant blue staining of fractionated material. Proteins were extracted from promastigotes of V121 (lanes 1 to 4) or L119 (lane 5). Lanes: 1, total lysate (20 μl); 2, insoluble material after TX-114 lysis; 3, aqueous phase (20 μl); 4, TX-114 detergent phase (8 μl); 5, TX-114 detergent phase (8 μl). (B) Lactoperoxidase-catalyzed iodination of promastigotes from V121, followed by TX-114 fractionation. Lanes: 1, total lysate (10 μl); 2, insoluble material after TX-114 lysis (5 μl); 3, insoluble material after TX-114 lysis (10 μl); 4, TX-114 detergent phase (10 μl); 5, aqueous phase (15 μl); 6, aqueous phase (5 μl). The positions of the molecular weight standards (M) are shown beside each panel as follows: phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), and carbonic anhydrase (30,000).

Dot blot. Purified *L. major* glycophospholipids (26a, 27) were dotted onto a nylon membrane (Zeta probe; Bio-Rad Laboratories, Richmond, Calif.) at a concentration of 2 μg/ml in PBS. The membrane was incubated in 5% skim milk in PBS as for Western blots and probed with mouse sera at a dilution of 1:100 as described above. Immune complexes were detected with radioiodinated protein A as in the Western blots.

RESULTS

SDS-PAGE profiles of TX-114 phase-separated proteins from *L. major*. The protein pattern obtained following TX-114 phase separation of stationary-phase promastigotes of V121 is shown in Fig. 1A. Lane 1 shows the Coomassie blue R250-stained profile of the whole-cell lysate, and lane 2 shows the pattern of material insoluble in TX-114. The two major bands migrating with approximate M_r 50,000 to 55,000 in lane 2 are probably cytoskeletal proteins, as shown

previously (12). The protein profiles of the aqueous phase (lane 3) and the TX-114 phase (lane 4) show clear differences compared with the profile of the whole-cell lysate (lane 1). The TX-114 phase, which includes most integral membrane proteins, shows a clear enrichment for some proteins which are depleted from the aqueous phase. This TX-114 phase includes at least 40 bands weakly stained with Coomassie blue. The protein pattern in the TX-114 phase of promastigotes of strain L119 is shown in lane 5 for comparison to the profile of V121 promastigotes (lane 4). The profile shows a striking increase in the staining intensity of a group of polypeptide bands in L119 with an approximate M_r of 54,000 to 63,000. In addition, there were differences in the lower-molecular-weight species as well as an absence of polypeptides detectable by Coomassie blue staining above M_r 80,000 in strain L119 (lane 5).

To determine if any of the TX-114 phase proteins seen in strain V121 (Fig. 1A, lane 4) were externally oriented on the parasite surface, lactoperoxidase-catalyzed iodination of promastigote surface proteins was performed, followed by TX-114 phase separation. As shown in Fig. 1B, the total-cell lysate (lane 1) shows one major iodinated polypeptide migrating at approximately M_r 58,000 which is enriched into the TX-114 phase (lane 4) along with at least nine other minor polypeptide bands (lane 4). This M_r-58,000 band probably represents the gp63 of V121 (3), which is the dominant surface-labeled protein in *L. major* (see below). In this experiment, the TX-114 phase was not concentrated by methanol precipitation (see Materials and Methods), and the amount loaded in lane 4 represents approximately 1% of the total proteins in lane 1. Several minor polypeptide bands were apparent in the TX-114-insoluble fraction (lanes 2 and 3). The major band in this phase corresponds to the dominant M_r-58,000 band in the TX-114 phase and probably represents incomplete separation of this molecule into the TX-114 phase during the extraction procedure or spillover during loading of the sample onto the gel. The minor M_r-58,000 polypeptide doublet partitioning in the aqueous phase (lanes 5 and 6) may represent the water-soluble form of gp63 released during the lengthy sample preparation (3). The absence of significant labeling of the aqueous-phase proteins suggests that very little (if any) labeling of internal components occurred during the surface iodination procedure. The iodination pattern of surface proteins of strain L119 promastigotes was similar to that shown for V121 (data not shown). These results show that a proportion of the total proteins which partition into the TX-114 detergent phase are externally oriented on the promastigote surface. These results also show that all radioiodinated surface proteins partition into the detergent phase and are thus integral membrane proteins as expected.

Immunoprecipitation of TX-114 phase material. Immunoprecipitations of TX-114 phase polypeptides from surface-iodinated parasites using a panel of different sera revealed common antigenic patterns. As shown in Fig. 2, immunoprecipitation with control mouse (lane 1), human (lane 2), or rabbit (lanes 3 and 8) serum indicated only a minor degree of background reactivity with the dominant surface-iodinated protein of *L. major* (lane 3). Rabbit anti-V121 promastigote (Fig. 2, lane 5) and mouse antibodies to parasite glycoproteins which bind ConA (Fig. 2, lane 4) immunoprecipitated identical patterns of radiolabeled proteins. The major M_r-58,000 labeled band in V121 is precipitated with both sera along with three other bands of approximate M_r 94,000, 89,000, and 42,000 and two very faint bands of approximate M_r 105,000 and 81,000 (Fig. 2., lanes 4 and 5). A similar

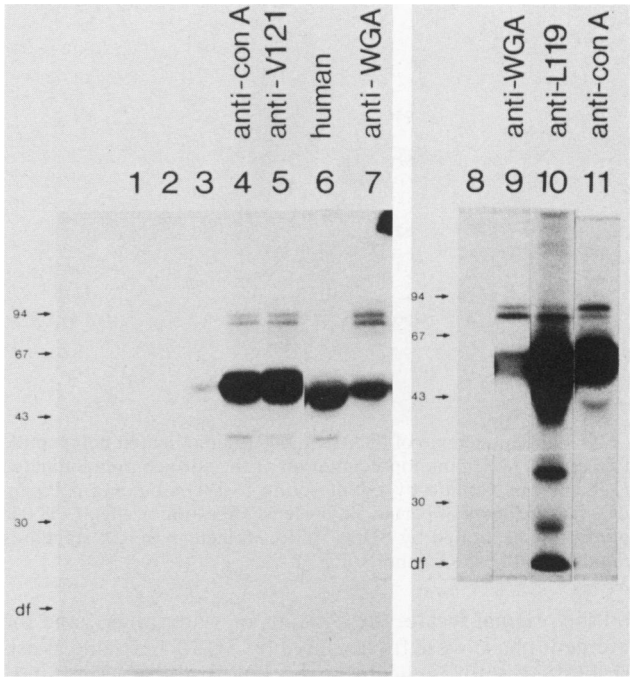

FIG. 2. Immunoprecipitation profiles of surface-radioiodinated material from promastigotes of *L. major*. Immunoprecipitation of TX-114 phase material from V121 (lanes 1 to 7) and total lysates of L119 (lanes 8 to 11) is shown for comparison. Lanes 1, normal mouse serum; 2, normal human serum; 3 and 8, normal rabbit serum; 4 and 11, rabbit anti-ConA-binding material; 5, rabbit anti-V121 promastigote serum; 6, pool of human sera from visceral leishmaniasis patients in Brazil; 7 and 9, rabbit anti-WGA-binding material; 10, rabbit anti-L119 promastigote serum. Molecular weight standards are as described in the legend to Fig. 1. df, Migration of the dye front.

pattern is seen with serum to WGA-binding material (Fig. 2, lane 7), with very weak recognition of the M_r-42,000 band. A pool of human sera from several Brazilian kala-azar patients did not recognize any of the higher-molecular-weight species but precipitated the M_r-58,000 and -42,000 bands (Fig. 2, lane 6).

The recognition pattern of surface-iodinated lysates of LRC-119 promastigotes using rabbit antibodies to promastigote glycoproteins which bind ConA or WGA was very similar to the pattern seen with TX-114 phase material from V121, with some variation in the relative abundance of the high-molecular-weight antigens (Fig. 2, lane 9 versus lane 7, lane 11 versus lane 4). Anti-L119 promastigote serum (Fig. 2, lane 10) precipitated the same pattern of proteins as the anti-glycoprotein sera, with the addition of two low-molecular-weight bands of M_r 34,000 and 27,000.

To determine whether the major M_r-58,000 radioiodinated polypeptide recognized by the sera described in Fig. 2 is the well-characterized gp63 (for a review, see reference 3), the band was cut out of the gel (Fig. 3A) and subjected to limited proteolysis by using staphylococcal V8 protease (9, 15, 17). The M_r-58,000 polypeptide displays a very similar peptide map (Fig. 3B) to that described for gp63 (15), indicating that this is indeed gp63.

These results show that a proportion of the surface-iodinated TX-114 phase proteins are WGA- and ConA-binding glycoproteins.

Immunoblotting of TX-114 phase material. Lysates of LRC-119 promastigotes were phase separated in TX-114,

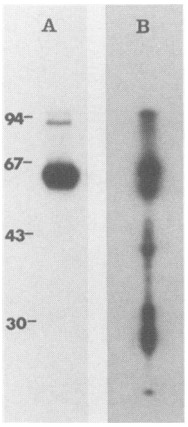

FIG. 3. Peptide map of the M_r-58,000 radioiodinated polypeptide of *L. major*. (A) Immunoprecipitation of the surface-radioiodinated M_r-58,000 polypeptide by rabbit serum to WGA-binding polypeptides. (B) Products of partial V8 protease digestion of the M_r-58,000 polypeptide separated by SDS-PAGE. Molecular weight standards are described in the legend to Fig. 1.

and the original lysate, the aqueous or water phase, and the detergent phase were fractionated by SDS-PAGE and Western blotted onto duplicate nitrocellulose filters. The filters were then probed with rabbit antibodies to *L. major* WGA-binding proteins (Fig. 4, WGA) or ConA-binding proteins (Fig. 4, ConA). Only a minority of the promastigote glycoproteins could be detected in the TX-114 phase. The TX-114 phase was enriched for two major ConA-binding glycoproteins of M_r 94,000 and 92,000 as well as an M_r-63,000 band, probably gp63. These may be the same polypeptides immunoprecipitated by the same antibodies in Fig. 2. Four additional minor bands in the lower-molecular-weight range between M_r 40,000 and 35,000 were also apparent. A number of ConA-binding glycoproteins were present in the water phase as well as the original lysate but not detectable in the TX-114 phase.

The abundance of the WGA-binding proteins in the TX-114 phase was about 50-fold lower than that of the ConA-binding proteins. Only a faint band of M_r 90,000 was visible when the same amount of TX-114 phase protein was loaded in the gel and probed with the rabbit anti-WGA-binding serum (Fig. 4, TX 114 a). After methanol precipitation and concentration 50-fold, however, two dominant bands of M_r 94,000 and 92,000 became apparent in the TX-114 phase, in addition to a few minor, lower-molecular-weight glycoproteins (Fig. 4, TX 114 b). Several WGA-binding glycoproteins detectable in the original lysate were extracted into the water phase and not detected in the TX-114 phase. These results show that several ConA-binding glycoproteins but few WGA-binding glycoproteins of *L. major* are integral membrane proteins. In contrast to the immunoprecipitation of radioiodinated proteins by the antibodies to WGA-binding proteins (Fig. 2, lane 7), gp63 was not detected on the Western blot (Fig. 4, TX 114 b).

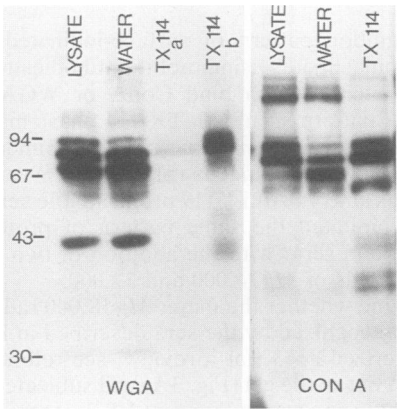

FIG. 4. Immunoblotting analysis of antigens of *L. major* LRC-L119 phase separated by TX-114 fractionation. Antigens were detected on an immunoblot by rabbit antibodies raised to ConA- or WGA-binding material as described in Materials and Methods. Antigens were detected in the original lysate, the water phase, and the detergent phase (TX 114 a), adjusted to their concentrations in the original lysate or in the detergent phase, which had been concentrated 50-fold (TX 114 b). Molecular weight standards are as described in the legend to Fig. 1.

Vaccination of BALB/c *H-2*^{k} mice with TX-114 phase membrane proteins. To assess if proteins in the TX-114 phase of *L. major* can protect BALB/c *H-2*^{k} mice against infection with *L. major*, TX-114 phase proteins from L119 promastigotes were prepared and used in vaccination experiments. L119 was used as the source of TX-114 phase proteins since this parasite has been shown to express undetectable levels of LPG, a known protective molecule (19), and injection of live avirulent L119 organisms has been shown to successfully vaccinate both genetically resistant and susceptible mice (28).

In two experiments shown in Fig. 5, control mice injected with *C. parvum* alone developed lesions 3 to 4 weeks after infection. Only a few mice vaccinated with the TX-114 phase material and *C. parvum* developed lesions, and the lesions that did develop were smaller and their size was maintained for up to 8 weeks after challenge (Fig. 5). In one experiment in which mice were vaccinated with proteinase K-treated TX-114 phase material, no protection was observed (data not shown).

To examine the surface membrane protein antigens recognized by the vaccinated mice, serum was collected 1 day before challenge with virulent promastigotes of *L. major* V121 and 4 weeks after challenge. Figure 6 (left panel) shows immunoprecipitation profiles of lysates of surface-radiolabeled promastigotes of *L. major* L119 with serum from control or vaccinated mice before and after challenge. Mice injected with *C. parvum* alone (Fig. 6, control) or with protease-treated material (Fig. 6, protease) did not recognize any radioiodinated antigens. Mice vaccinated with the TX-114 phase material recognized a major surface-labeled protein of approximate M_r 63,000, probably gp63, together with a minor band of approximate M_r 80,000 (Fig. 6, TX 114 [left]). Following challenge with V121, two additional polypeptides of approximate M_r 89,000 to 94,000 were also recognized (Fig. 6, TX-114 [right]). Serum from the control infected mice immunoprecipitated a similar protein pattern except that the M_r-89,000 protein was absent (not shown).

To ascertain that the vaccinating effect observed with the TX-114 phase material was not due to contaminating glycolipids, such as LPG (27) or glycoinositol phospholipid (GIPL) (13, 26a), sera from the vaccinated mice were tested for binding to these glycolipids. The vaccinated mice had no detectable antibodies to either LPG or GIPL (Fig. 6, right panel, TX 114). Following challenge with live V121 organisms, however, the immune mouse serum recognized GIPL but probably not LPG (Fig. 6, left panel, immune). The preparation of LPG used in this experiment was contaminated with small amounts of GIPL. This is demonstrated by the fact that the anti-GIPL monoclonal antibody L-5-34,

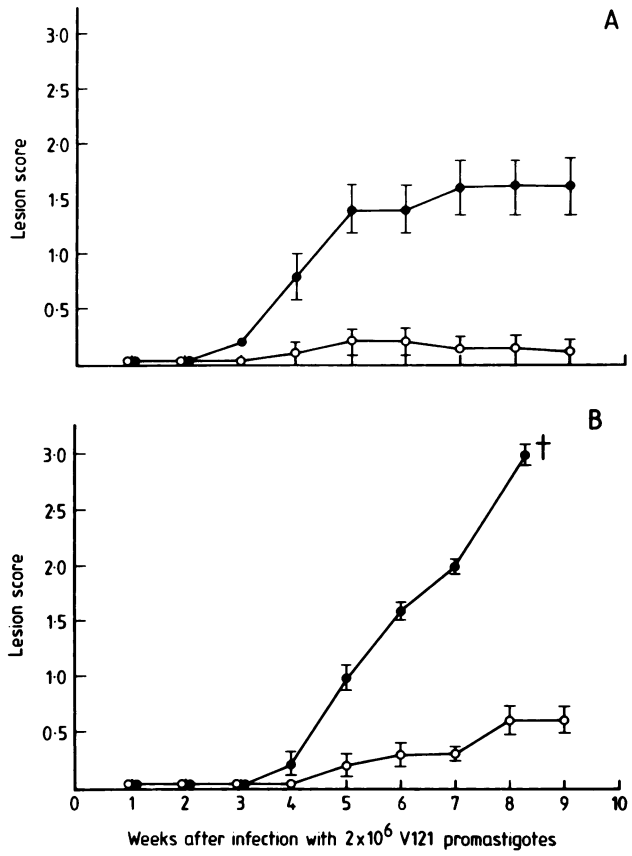

FIG. 5. Vaccination of mice with TX-114 phase membrane proteins. Development of lesions in BALB/c *H-2*^{k} mice immunized with the TX-114 phase material isolated from promastigotes of LRC-L119 with *C. parvum* as adjuvant (○) or adjuvant alone (●) and challenged with 2×10^6 live *L. major* promastigotes was compared. Data are expressed as the lesion score plus or minus the standard error of the mean.

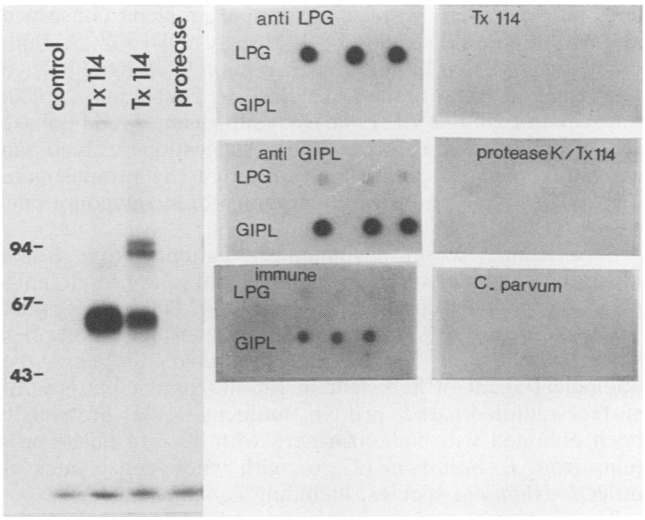

FIG. 6. Detection of radiolabeled surface protein (left panel) and glycolipid antigens (right panel) by antibodies from mice vaccinated with the TX-114 phase-separated material from LRC-L119 before and after challenge with live *L. major* V121 promastigotes. Left panel: Radioiodinated LRC-L119 lysate was immunoprecipitated with control serum from mice injected with *C. parvum* alone (control) or serum from vaccinated mice (TX114, left lane) before challenge infection and with serum from the immune mice after infection (TX114, right lane), as well as serum from mice immunized with the TX-114 detergent phase which was treated with proteinase K (protease). Right panel: Preparations of LPG or GIPL were dotted in triplicate onto a nitrocellulose filter and probed with sera from mice vaccinated with proteinase K-treated TX-114 phase material (protease K/TX114), *C. parvum* alone (*C. parvum*), or TX-114 phase material plus *C. parvum* (TX 114). Other filters were probed with monoclonal antibody WIC-79.3 to LPG (anti LPG), monoclonal antibody L-5-34 to GIPL (anti GIPL), or serum from mice vaccinated with TX-114 phase material plus *C. parvum* and challenged with live promastigotes of *L. major* V121 (immune).

which has been shown not to recognize LPG (13), bound the LPG dotted on this filter (Fig. 6, right panel, anti-GIPL). Sera from control mice vaccinated with *C. parvum* alone or proteinase K-treated TX-114 phase material also did not recognize either LPG or GIPL (Fig. 6).

It appears, therefore, that the integral membrane proteins of promastigotes of *L. major* L119 that partition into the TX-114 phase could protect mice from disease caused by the virulent *L. major* V121.

DISCUSSION

In this study, the integral membrane proteins of stationary-phase promastigotes of *L. major* have been separated into the nonionic detergent TX-114 and characterized by using surface radioiodination, Western blotting, and immunoprecipitation with a panel of antisera. Using the TX-114 fractionation procedure of Bordier (2), we have identified a minimum of 40 integral membrane proteins, approximately 10 of which are surface oriented as determined by surface radioiodination. Western blot and immunoprecipitation analysis of the TX-114 phase proteins using serum to ConA- or WGA-binding glycoproteins revealed a relatively simple profile of approximately 10 major integral membrane glycoproteins, 6 of which are surface oriented.

The TX-114 phase separation procedure has been applied successfully to *Plasmodium falciparum* (34), *Schistosoma japonicum* (32), *Trypanosoma brucei gambiense* (1), and *L. donovani* (22). The method used in this study is a modification of that of Smythe et al. (34), who improved the efficiency of solubilization of the membranes compared with the original method of Bordier (2). We have successfully applied this technique to *L. major* as shown by the different and specific Coomassie blue-stained profiles of each phase and by the fact that all radiolabeled proteins from surface-radioiodinated promastigotes partitioned into the detergent phase. The pattern of surface-oriented integral membrane proteins identified by radioiodination is quite simple in *L. major*. The dominant iodinated protein of *L. major*, gp63, observed in all *Leishmania* species studied so far (3, 4, 10, 15, 16, 26) is prominent in both V121 and L119 protein profiles as an M_r-58,000 to -63,000 band. gp63 partitions into the TX-114 phase by virtue of its lipid anchor (14). At least nine other minor polypeptides were identified as surface-oriented integral membrane proteins. The approximately 20-fold enrichment for integral membrane proteins obtained by using the TX-114 phase separation procedure has allowed us to identify five minor surface-oriented glycoproteins, apart from gp63, in *L. major* V121. These molecules of approximate M_r 105,000, 94,000, 89,000, 81,000, and 42,000 are ConA- or WGA-binding glycoproteins. The group of proteins of M_r 94,000, 89,000, and 81,000 identified in *L. major* are of interest since they are recognized by sera from cutaneous leishmaniasis patients from Israel and Venezuela

and may represent surface membrane antigens conserved among *Leishmania* species (P. J. Murray and T. W. Spithill, unpublished data). In addition, *L. major* L119 exhibits two additional surface-oriented proteins (M_r 34,000 and 27,000) not seen in *L. major* V121. These results using TX-114 phase separation are in agreement with the suggestion by Handman et al. (20) and Gardiner et al. (16) that the promastigote displays a simple profile of integral membrane glycoproteins on its surface.

Sera from visceral leishmaniasis patients from Brazil identified only two surface integral membrane protein antigens, the gp58-63 complex and an M_r-42,000 polypeptide. These antigens represent conserved surface molecules shared between *L. major* and *L. donovani chagasi*, the etiological agent of kala-azar in Brazil. Similar patterns of surface-radioiodinated protein antigens have previously been obtained with kala-azar sera with TX-114 phase proteins from *L. donovani* (22) or with whole-cell lysates of other *Leishmania* species, including *L. major* (10, 26).

The comparison of the integral membrane profiles of two strains of *L. major* (V121 and L119) revealed a striking difference in the expression of the gp63 complex. The Coomassie blue-stained protein profiles of total (whole-cell) detergent lysates of V121 and L119 are similar when analyzed by SDS-PAGE (data not shown). TX-114 fractionation of integral membrane proteins from V121 and L119, however, revealed that these strains differ in their membrane protein profiles as detected by Coomassie blue staining (Fig. 1A). Most notable is the appearance in L119 of three prominent bands migrating at M_r 54,000 to 63,000 that were much less abundant in V121. In order to compare these strains, immunoblots and immunoprecipitations were performed with antisera to glycoproteins. It appears that V121 and L119 differ in the abundance of the major M_r-58,000 band. The set of three bands at M_r 54,000 to 63,000 seen in the TX-114 detergent phase of L119 probably reflects differences in glycosylation states of a single protein since immunoprecipitation with rabbit antiserum to WGA-binding glycoproteins and human sera immunoprecipitated the lower-molecular-weight species, while rabbit serum to ConA-binding glycoproteins precipitated the whole complex. We conclude that the major group of bands of M_r 54,000 to 63,000 seen in the detergent phase of L119 and the major iodinatable species of M_r 58,000 in V121 are the same and represent the gp63 of these strains.

The presence of a major M_r-63,000 iodinatable protein (gp63) has been described in all *Leishmania* species, and its molecular nature has been studied in detail (3). The gene for this integral membrane protein has recently been cloned and sequenced (7). Bouvier et al. (5) concluded that in *L. major* LEM513, gp63 is present at 0.5 to 1% of the total promastigote protein in a copy number of 5.0×10^5 per cell. In this study, we have shown that the major surface protein is present at much higher abundance in the avirulent strain L119 than in the virulent clone V121. Similar variation between strains in the abundance of gp63 has been described in other *Leishmania* species (4, 22). *L. major* L119 has been shown to be deficient in the expression of LPG on its surface and is avirulent in mice (21). The fact that V121 is highly infective for mice while L119 is not suggests that other molecules in addition to gp63 play a role in parasite infectivity. In this respect, it is interesting to speculate on the importance of LPG, since there seems to be a reciprocal relationship between the abundance of LPG and gp63 in the virulent V121 compared with the avirulent L119 (21).

Despite the lack of LPG, a recognized host-protective antigen, in L119 promastigotes, these organisms can vaccinate and protect mice from infection with the virulent cloned line V121 (28). This suggested that antigens other than LPG could mediate protection. This study examined the value of promastigote integral membrane proteins as possible vaccine candidates and showed that significant protection was achieved with as little as 400 μg of total membrane proteins. Antisera from vaccinated mice recognized gp63 as well as a minor surface-oriented antigen of M_r 80,000. Since gp63 is the most prominent membrane protein antigen in L119, it is possible that it contributes significantly to the vaccination effect observed, although a role for other proteins recognized by T cells but not detected by immunoprecipitation cannot be excluded. This is in agreement with studies by Russell and Alexander (33) demonstrating only a partial protection of BALB/c mice immunized with gp63 in liposomes. We also observed some variability in the protection obtained (Fig. 5); it was complete in one experiment and partial in a second experiment. In the second experiment, however, the lesions in the vaccinated mice remained less than 5 mm in diameter for more than 15 weeks while the control mice had large lesions and had to be killed at 8 weeks (data not shown). The variability in the protection data was mirrored by the variability in the size and speed of lesion development in the control mice, a well-described problem in murine cutaneous leishmaniasis (29). In addition, Nogueira et al. (30) have shown that serum from mice immunized with heat-killed or γ-irradiated promastigotes of *L. major* recognizes gp63 as well as several other surface-labeled proteins. The identification of integral membrane protein antigens, apart from gp63, which are recognized by immune T cells is clearly warranted.

This study has identified a relatively simple profile of surface-oriented integral membrane proteins. These will be assessed as potential components of a cocktail subunit vaccine comprising integral membrane proteins, possibly inserted in liposomes prepared from *Leishmania* host-protective glycolipids.

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