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CHARACTERIZATION OF A MEMBRANE ANTIGEN OF *Leishmania amazonensis* THAT STIMULATES HUMAN IMMUNE RESPONSES[1]

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To investigate human immune responses to defined leishmania Ag we have begun to characterize biochemically and immunologically, an abundant 42-kDa surface Ag of *Leishmania amazonensis*, a causative agent of human leishmaniasis. We have shown that this Ag, La gp42, is expressed on the surface of *L. amazonensis* promastigotes, being anchored to the membrane by a glycosyl-phosphatidylinositol moiety. As demonstrated by lectin blotting studies, La gp42 is glycosylated, binding both Con A and wheat germ agglutinin. Immunologically, La gp42 is strongly recognized by sera from patients with different forms of leishmaniasis as well as by patients with Chagas' disease. In addition, we show that purified La gp42 stimulates the proliferation of human T lymphocytes obtained from several leishmaniasis patients. Finally, the N-terminal sequence of La gp42 was obtained and a serologically cross-reactive 42-kDa protein with a homologous sequence was identified in *Leishmania major*.

Leishmania are obligate intracellular parasites of macrophages. They cause a spectrum of human disease ranging from self-healing cutaneous lesions to diffuse cutaneous and mucosal manifestations or disseminated and often fatal visceral leishmaniasis. *Leishmania* undergo morphologic and biochemical changes in both the sandfly vector and vertebrate host, contributing to the antigenic diversity of these organisms. Host immune responses are usually strong, with recovery and resistance to reinfection dependent on the development of appropriate T lymphocyte responses (1, 2).

There is considerable interest in the characterization of host responses to leishmania Ag for the development of an effective immunization protocol as well as for a better understanding of pathogenesis. Humans, as well as experimental animals, are readily protected against leishmaniasis by active immunization (3). The identification of parasite Ag eliciting protective responses and cytokine production is crucial, particularly in light of studies in mice that have associated certain T cell phenotypes and cytokine profiles with protective effects (4–6). However, limited information is available on specific leishmania Ag that stimulate T lymphocyte responses, with most of these studies confined to the investigation of murine immune responses.

Recent efforts have turned toward the identification of Ag recognized by T cells in human leishmaniasis. Using nitrocellulose-immobilized *Leishmania donovani* Ag separated by one- and two-dimensional gel electrophoresis, Melby and co-workers (7, 8) revealed the heterogeneity of T cell responses of patients in various disease states to specific leishmania Ag. Using purified Ag selected by their reactivity with patient serum, Reed et al. (9) identified two *Leishmania chagasi* glycoproteins of 30 and 42 kDa that consistently elicited high proliferative responses as well as IL-2 and IFN-γ production from T lymphocytes of leishmaniasis and Chagas' disease patients. We report the isolation of and human immune responses to a 42-kDa surface glycoprotein of *Leishmania amazonensis* promastigotes.

MATERIALS AND METHODS

Parasites and culture. L. amazonensis (MHOM/BR/84/BA32c5) was cloned by limiting dilution. Promastigotes of both *L. amazonensis* and *Leishmania major* (obtained from David Moser, Temple University, Philadelphia, PA) were cultured in medium 199 (GIBCO, Grand Island, NY) supplemented with 10% Serum Plus (Hazelton Biologics, Lenexa, KS). *L. chagasi* (MHOM/BR/82/BA-2.C1) promastigotes were cultured as previously described (9). *L. amazonensis* amastigotes were grown in BALB/c ByJ (The Jackson Laboratories, Bar Harbor, ME) mouse footpads and purified by homogenizing the infected tissue in PBS-G[3] and passing the suspension through a 20-gauge animal intubation needle (Popper and Sons, Inc., New Hyde Park, NY) four times to disrupt the host cells. Cell debris was removed by two low-speed centrifugations (200 × *g* for 10 min) and amastigotes pelleted (2100 × *g* for 20 min) and washed several times with PBS-G.

Patient cells and sera. Cells and sera were obtained from well defined patient groups from our study areas as previously described (9–11). Differential diagnoses were made based on clinical evaluation (10, 11), serologic testing (12), intradermal skin testing (13), and parasite isolation and identification. Pooled sera from patients with documented infection with *L. amazonensis*, *L. major*, *L. chagasi*, *L. donovani*, and *Trypanosoma cruzi* were prepared. Non-endemic area normal sera were used as controls.

Membrane preparation and PI-PLC cleavage. Triton X-114 solubilization and phase separation of parasite membrane proteins were carried out as previously described (14), with modifications. Approximately 2 to 3 × 10^{10} late-log phase promastigotes of *L.*

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[3] Abbreviations used in this paper: PBS-G, PBS + 2% glucose; PBS-T, PBS + 0.1% Tween 20; PI-PLC, phosphatidylinositol specific-phospholipase C; GPI, glycosyl-phosphatidylinositol; La gp42, 42-kDa glycoprotein of *L. amazonensis*; Lm gp42, 42-kDa glycoprotein of *L. major*; LPG, lipophosphoglycan.

amazonensis or *L. major* were resuspended in hypotonic lysis buffer (42.5 mM KCl, 5 mM $MgCl_2$) and subjected to five freeze/thaw cycles. A membrane-enriched pellet was obtained after centrifugation at 2000 × *g* for 15 min at 4°C, resuspended in 5 ml of 10 mM Tris, pH 7.4, 140 mM NaCl, 5% Triton X-114 (Pierce Chemical Co., Rockford, IL), and incubated for 15 min on ice. The Triton X-114-solubilized material was then warmed to 32 to 35°C that permitted a temperature-dependent phase separation, with integral membrane proteins fractionating into the detergent phase. Aqueous and detergent phases were separated by centrifugation (1600 × *g* for 10 min) at 30°C through a 1-ml 6% sucrose pad. The detergent phase was diluted to 5 ml and chilled for 10 min on ice. Five U of *Bacillus thuringiensis* PI-PLC (American Radiolabeled Chemicals Inc., St. Louis, MO) were added to this detergent soluble fraction and incubation continued at 35°C for 1 h. Detergent and aqueous phases were again separated as above, with PI-PLC-cleaved GPI-anchored membrane proteins fractionating in the aqueous phase.

Labeling and identification of surface proteins. L. amazonensis Ag were metabolically labeled with ^{35}S-methionine as described (9). Cell surface proteins were biotinylated according to the method of Hare and Lee (15). Briefly, 2 × 10^8 promastigotes from mid- to late-log phase cultures were pelleted by centrifugation at 2100 × *g* for 20 min, washed twice with PBS-G, and resuspended in 10 ml of methionine-free RPMI 1640 containing 1% BSA. ^{35}S-methionine (1000 Ci/mmol; NEN Research Products, Boston, MA) was added at 10 μCi/ml and cells were cultured for 16 h at 25°C. After this incubation, biosynthetically labeled cells were washed twice in PBS-G. Parasites were then resuspended in PBS containing 0.4 mg/ml NHS-S-S-Biotin (Pierce) and incubated at 37°C for 15 min, followed by 15 min at 4°C. The cells were washed to remove free biotin and pellets were stored at −80°C until use.

Labeled *L. amazonensis* promastigotes were resuspended in hypotonic lysis buffer and PI-PLC-cleavable membrane proteins prepared as described. Streptavidin-agarose beads (Sigma Chemical Co., St. Louis, MO) were added to this fraction to bind biotinylated proteins. Incubation continued overnight at 4°C with end-over-end rotation. The beads were collected by centrifugation and washed three times in 1 × wash buffer (PBS + 1% Triton X-100), once in 1 × wash buffer containing 1 M NaCl, once with 0.1 × wash buffer, and once in 1 × wash buffer containing 0.1% SDS. Precipitated parasite proteins were liberated from the streptavidin-agarose beads by boiling for 2 min in 25 μl SDS sample buffer containing 2% 2-ME. Surface proteins were separated by SDS-PAGE on 12% gels according to Laemmli (16) and visualized by autoradiography. Metabolically labeled, non-biotinylated *L. amazonensis* promastigotes processed concurrently served as specificity controls.

Parasite Ag preparations. Soluble *L. amazonensis* and *L. chagasi* lysates were prepared from stationary phase parasite cultures. Promastigotes were subjected to 10 rapid freeze/thaw cycles followed by centrifugation at 5000 × *g*. The soluble supernatant was aliquoted at 1 mg/ml and stored at −20°C until use.

La gp42 and La gp63 were purified from the PI-PLC-cleaved fraction of *L. amazonensis* membrane proteins by preparative IEF. The separation was carried out using the Bio-Rad Rotofor IEF cell and 1% 3/10 ampholytes (Bio-Rad Laboratories, Richmond, CA) to establish the pH gradient. Fractions containing either La gp42 or La gp63 were pooled and focused a second time using 0.5% 3/10 ampholytes. Peak fractions were pooled and dialyzed against 25 mM Tris-HCl, pH 8.0, 150 mM NaCl. Lm gp42 from the PI-PLC-cleaved fraction of *L. major* membrane proteins and Lc gp42 from a whole *L. chagasi* promastigote lysate were purified by preparative SDS-PAGE and electroelution as described (9). Protein concentration of the isolated material was determined using the Pierce BCA protein assay, and purity assessed by silver staining (Bio-Rad) after SDS-PAGE.

Immunoblot analyses. L. amazonensis cell membrane proteins cleaved with PI-PLC or purified La gp42 were separated by SDS-PAGE as above and transferred electrophoretically to nitrocellulose membranes as described previously (17). After blocking with PBS containing 5% FCS (Hazelton), individual strips were incubated for 30 min with sera diluted in PBS-T (1/200 for human sera). After three 10-min washes with PBS-T, the strips were incubated for 30 min with alkaline-phosphatase conjugated goat anti-human IgG + IgA + IgM (Zymed Laboratories Inc., San Francisco, CA) diluted 1/1000 in PBS-T. After three PBS-T washes, nitroblue tetrazolium (Sigma) and 5-bromo-4-chloro-3 indolyl phosphate (Sigma) were applied as substrate. Development was stopped after 40 min, and unreacted substrate removed from Ag strips by washing with dH_2O.

Immunoblots involving purified La gp42, Lm gp42, and Lc gp42 were carried out as above and probed with various rabbit sera at a 1/400 dilution. Bound antibody was detected using ^{125}I-protein A (1 × 10^6 cpm/blot), followed by autoradiography.

Lectin blot analyses. Purified La gp42 was separated by SDS-PAGE, transferred to nitrocellulose and blocked as above. Ag strips were incubated for 30 min with the following biotinylated lectins diluted 1/400 in PBS-T from 1 mg/ml stock solutions: *Arachis hypogaea* (peanut), *Canavalia ensiformis* (Con A), *Phaseolus vulgaris*, *Triticum vulgaris* (wheat germ) (Sigma). Blots were then washed three times in PBS-T and avidin-alkaline phosphatase (Zymed) applied for 30 min at a dilution of 1/1500, and subsequently developed as described.

Lymphocyte proliferation assays. PBMC from leishmaniasis patients and non-infected donors were obtained by separation on Ficoll-Paque (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) and stored frozen in liquid nitrogen. Cells were cultured in complete medium (consisting of RPMI 1640 (GIBCO) supplemented with L-glutamine, sodium pyruvate, gentamycin, 5 × 10^{-5} M 2-ME, and 10% screened human type A serum) at 4 × 10^5 cells/well in 96-well flat-bottom plates (Costar, Cambridge, MA), with or without Ag, in a total volume of 200 μl. After 5 days, 1 μCi/well of ^{3}H-thymidine (60 to 90 Ci/mmol; ICN Biomedicals, Inc., Costa Mesa, CA) was added, and radioactive incorporation determined after 18 h. In certain assays cell culture supernatants (50 to 100 μl) were harvested from wells before the addition of radionuclide. Supernatants were assayed for the presence of IFN-γ by a double sandwich ELISA using a mouse anti-human IFN-γ mAb (Chemicon, Temecula, CA) and a polyclonal rabbit anti-human IFN-γ antiserum. Human rIFN-γ (Genentech, Inc., South San Francisco, CA) was used to generate a standard curve ranging from 50 pg/ml to 1 ng/ml.

Production of rabbit antiserum against La gp42. An adult NZW rabbit (R & R Rabbitry, Stanwood, WA) was immunized with purified La gp42 for the production of a polyclonal antiserum. The immunization protocol consisted of two s.c. injections of 100 μg of purified protein given 4 wk apart. The first was administered in IFA (GIBCO) together with 500 ng of human rIL-1-β. The second was administered in IFA alone. Then 3 wk after the second immunization, rabbits were boosted with an i.v. injection of 40 μg of La gp42 and serum was collected 8 days later. Polyclonal rabbit antiserum was produced against the 42-kDa glycoprotein of *L. chagasi* using a similar immunization protocol as described previously (9).

N-terminal sequence analysis. PI-PLC-cleaved membrane preparations of *L. amazonensis* and *L. major* were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes according to the method of Matsudaira (18). For amino terminal sequencing, La gp42 and Lm gp42 were excised from the membrane and analyzed on an Applied Biosystems 477A Protein Sequencer with an on-line 120A HPLC (Applied Biosystems, Inc., Foster City, CA). Methods used for protein sequencing were according to the manufacturer's instructions.

RESULTS

Analysis of GPI-anchored membrane proteins of L. amazonensis and L. major. To begin analysis of surface Ag of *Leishmania, L. amazonensis*, and *L. major* promastigote membrane preparations were solubilized in Triton X-114. After a temperature-dependent phase separation, amphipathic integral membrane proteins were recovered in the detergent phase and the aqueous phase containing non-membrane-bound hydrophilic components was removed. This initial detergent phase was then treated with PI-PLC to release proteins anchored via a GPI moiety. After a second detergent phase separation, PI-PLC-cleaved membrane proteins previously fractionating in the detergent phase were recovered in the aqueous phase. Analysis by SDS-PAGE of *L. amazonensis* and *L. major* membrane proteins released by PI-PLC cleavage demonstrated the presence of a limited number of components ranging from 28 to 97 kDa (Fig. 1). Two major GPI-anchored membrane Ag of *L. amazonensis* were of interest (Fig. 1*A, lane 3*). The first, a doublet of approximately 61 to 63 kDa, was the major surface protease of leishmania, gp63, known to be GPI anchored. The second, was an abundant component of approximately 42 kDa. Similarly, gp63 and the 42-kDa component were released by PI-PLC cleavage from *L. major* promastigote membranes (Fig. 1*B, lane 2*). Although gp63 was clearly the most prominent Ag cleaved from *L. major* membranes, the 42-kDa membrane protein was

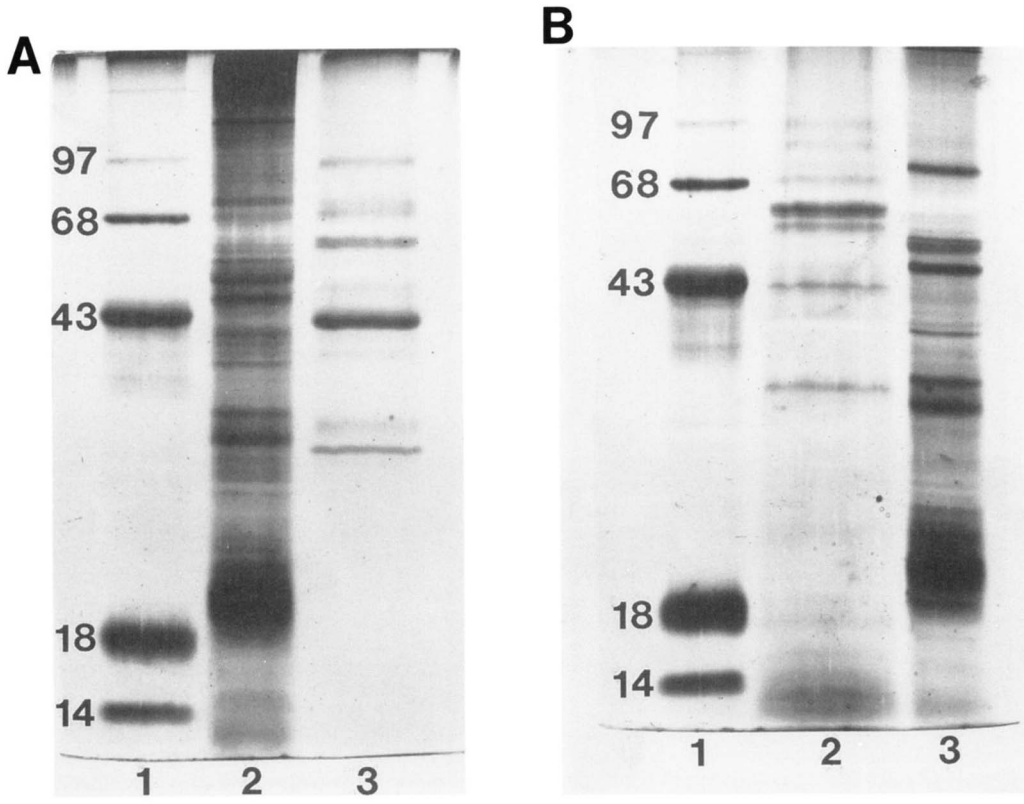

Figure 1. Silver stained SDS-PAGE of *L. amazonensis* (*A*) and *L. major* (*B*) membrane proteins released by PI-PLC cleavage. *A, Lane 1,* m.w. markers; *lane 2, L. amazonensis* promastigote lysate; *lane 3, L. amazonensis* PI-PLC released membrane proteins. *B, lanes 1,* m.w. markers; *lane 2, L. major* PI-PLC released membrane proteins; *lane 3, L. major* promastigote lysate.

approximately two- to threefold more abundant than gp63 in the *L. amazonensis* preparation.

Surface localization of La gp42. L. amazonensis promastigotes were metabolically labeled with ^{35}S-methionine and subsequently biotinylated to tag cell surface proteins. Throughout both the labeling and biotinylation procedure these parasite remained intact and viable. PI-PLC cleavable membrane proteins were prepared as above and biotinylated molecules precipitated with streptavidin-agarose beads, separated by SDS-PAGE, and visualized by autoradiography. Similar profiles of metabolically labeled PI-PLC cleaved membrane proteins were obtained from surface biotinylated *L. amazonensis* promastigotes (Fig. 2, *lane 1*), compared to non-biotinylated control cells (Fig. 2, *lane 2*). La gp42, and to a lesser degree La gp63, were specifically precipitated with streptavidin agarose beads from biotinylated membrane preparations (Fig. 2, *lane 3*). Neither molecule was present in the fraction containing non-specifically precipitated proteins from non-biotinylated control cells (Fig. 2, *lane 4*). These results were confirmed with *L. amazonensis* promastigotes surface labeled with ^{125}I. This demonstrated that in addition to gp63, an abundant 42-kDa, GPI-anchored Ag is expressed on the surface of *L. amazonensis* promastigotes.

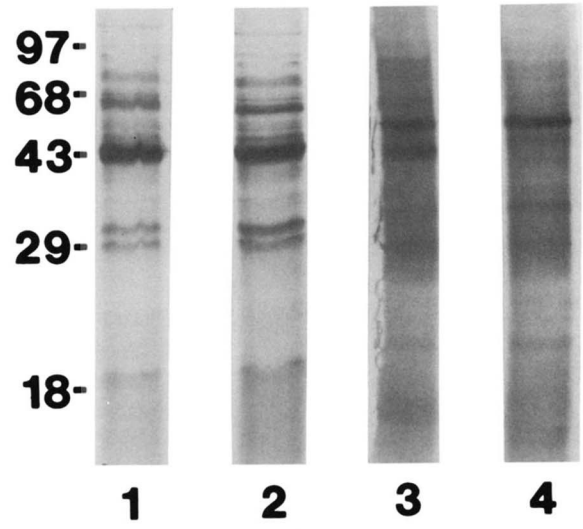

Figure 2. Surface localization of La gp42. Metabolically labeled PI-PLC cleaved membrane proteins were prepared from surface biotinylated (*lane 1*) *L. amazonensis* promastigotes and precipitated with streptavidin agarose beads (*lane 3*). For specificity controls, similar PI-PLC cleaved membrane proteins were prepared from non-biotinylated *L. amazonensis* promastigotes (*lane 2*) and precipitated with streptavidin agarose beads (*lane 4*). Labeled Ag were analyzed by SDS-PAGE followed by autoradiography.

Recognition of La gp42 by sera from patients with leishmaniasis and Chagas' disease. The serologic recognition of La gp42 was evaluated by immunoblot because of its surface expression and relative abundance. La gp42 was strongly recognized by sera from patients with *L. amazonensis* (Fig. 3, *lane 1*) or *L. major* (Fig. 3, *lane 2*) infection. Similarly, strong reactivity was observed with sera obtained from *L. donovani* and *L. chagasi* visceral leishmaniasis patients (Fig. 3, *lanes 3* and *4*). Serologic reactivity with La gp42 was not confined to leishmaniasis patients, inasmuch as Chagas' disease (*T. cruzi* infection) patient sera were equally reactive (Fig. 3, *lane 5*). No reactivity was observed with normal human sera (Fig. 3, *lanes 6*). Therefore, human antibody responses to La gp42 and the corresponding epitopes of other *Leishmania spp.* as well as *T. cruzi*, appear to be

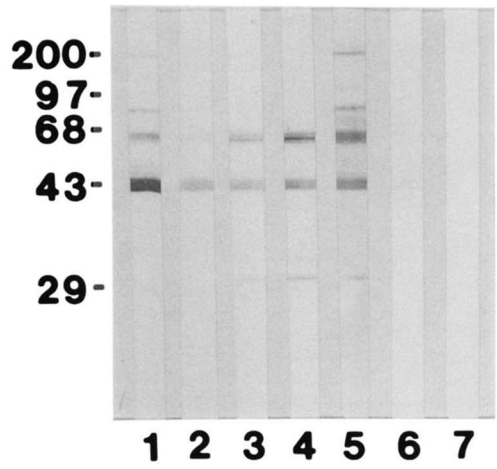

Figure 3. Immunoblot analysis of PI-PLC cleaved membrane Ag of *L. amazonensis* with leishmaniasis and Chagas' disease patient sera. Immunoblots were probed with pooled sera from patients infected with: *lane 1, L. amazonensis* ($n = 4$); *lane 2, L. major* ($n = 4$); *lane 3, L. donovani* ($n = 3$); *lane 4, L. chagasi* ($n = 4$); *lane 5, T. cruzi* ($n = 4$). Normal human sera ($n = 4$) and no primary antibody controls are shown in *lanes 6* and *7*, respectively.

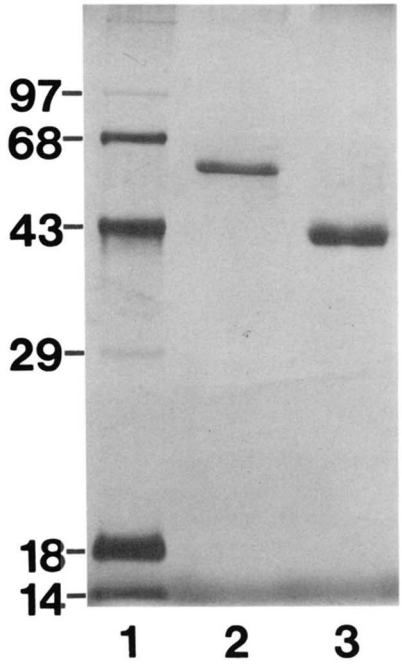

Figure 4. Silver stained SDS-PAGE of *L. amazonensis* membrane antigens purified by preparative isoelectric focusing. *Lane 1*, m.w. markers; *lane 2*, purified La gp63; *lane 3*, purified La gp42.

quite strong during infection with these parasites.

Analysis of carbohydrate content of purified La gp42. La gp63 and La gp42 were purified from *L. amazonensis* PI-PLC-cleaved membrane proteins by preparative IEF (Fig. 4, *lanes 2* and *3*). Approximately 300 μg of La gp63 and 750 μg of La gp42 were obtained from 2 to 3 $\times$ 10^{10} promastigotes. To ensure that the antigenicity of the purified La gp42 was maintained, immunoblots were performed with various patient sera. All sera shown to be reactive with La gp42 in PI-PLC-cleaved *L. amazonensis* membrane preparations (Fig. 3) remained strongly reactive with the purified molecule (Fig. 5, *lanes a* to *g*). Lectin binding studies were performed to analyze the carbohydrate content of La gp42. Con A and wheat germ agglutinin bound strongly to La gp42 (Fig. 5, *lanes h* and *i*), whereas peanut agglutinin and PHA were unreactive (Fig. 5, *lanes j* and *k*).

Patient proliferative responses to purified La gp42. To evaluate immunologic recognition by T cells, purified La gp42 (Fig. 4, *lane 3*) was added to PBMC from leishmaniasis patients, and proliferative responses compared to those obtained with *L. amazonensis* lysate (Fig. 1*A*, *lane 2*). Cells from all patients proliferated in response to parasite lysate, and five of the six patients responded to La gp42 (Table I). PBMC from several patients secreted IFN-γ in response to stimulation with La gp42. The concentrations of IFN-γ produced ranged from 100 to 200 pg/ml and represented approximately 10% of the amount of IFN-γ produced when the cells were stimulated with *L. amazonensis* lysate. La gp42 did not elicit proliferative responses or IFN-γ production by PBMC from uninfected individuals. Of interest were the particularly strong responses obtained in two individuals, L.V., a mucosal patient with extensive tissue destruction and J.M., a patient with active cutaneous disease. These results may indicate that T lymphocyte responses to La gp42 are strongest during active infection.

Reactivities of rabbit antisera raised against La gp42 and Lc gp42. Previous studies in this laboratory identified a glycosylated 42-kDa Ag of *L. chagasi* that stimulated proliferation of and IFN-γ secretion by T cells obtained from leishmaniasis and Chagas' disease patients. To assess the relationship between this *L. chagasi* Ag and the 42-kDa Ag of *L. amazonensis* and *L. major*, a monospecific rabbit sera was raised against La gp42 and tested for reactivity against purified La gp42, Lm gp42, and Lc gp42 by immunoblot analysis. As expected, there was strong reactivity of the rabbit anti-La gp42 sera on La gp42 (Fig. 6*A*, *lane 1*). Strong reactivity of this antiserum was also observed with the *L. major* 42-kDa membrane Ag (Fig. 6*A*, *lane 2*). However, no reactivity of the rabbit anti-La gp42 sera on Lc gp42 was observed (Fig. 6*A*, *lane 3*). A second rabbit antisera raised against Lc gp42 was similarly tested. As with the rabbit anti-La gp42 sera, no serologic cross-reactivity between Lc gp42 and La gp42 could be demonstrated using the rabbit anti-Lc gp42 sera (Fig. 6*B*, *lanes 1* and *3*). Of interest, reactivity of the anti-Lc gp42 sera was observed with the Lm gp42 (Fig. 6*B*, *lane 2*). Normal rabbit sera was not reactive with any of the three molecules (Fig. 6*C*, *lanes 1* to *3*). These data indicate that La gp42 and Lc gp42 appear to be serologically unrelated but that these Ag share some B cell determinants with the 42-kDa Ag of *L. major*.

Identification of homologous surface Ag of L. major promastigotes. Preliminary evidence from PI-PLC cleavage of parasite membrane preparations indicated the presence of an Ag of *L. major* promastigotes, which was similar or identical to La gp42 (Fig. 1). Serologic cross-reactivity was observed between the two Ag using the rabbit sera raised against La gp42. The relationship between the two Ag at the protein sequence level was investigated. PI-PLC-cleaved membrane proteins were prepared from *L. amazonensis* and *L. major* promastigotes, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes. The N-terminal sequences of the 42-kDa membrane proteins of both parasites were determined and are shown in Figure 7. A total of 32 contiguous N-terminal residues was assigned unambiguously for La gp42. Of the N-terminal 28 residues determined for Lm gp42, 26 of 28 were identical to those of

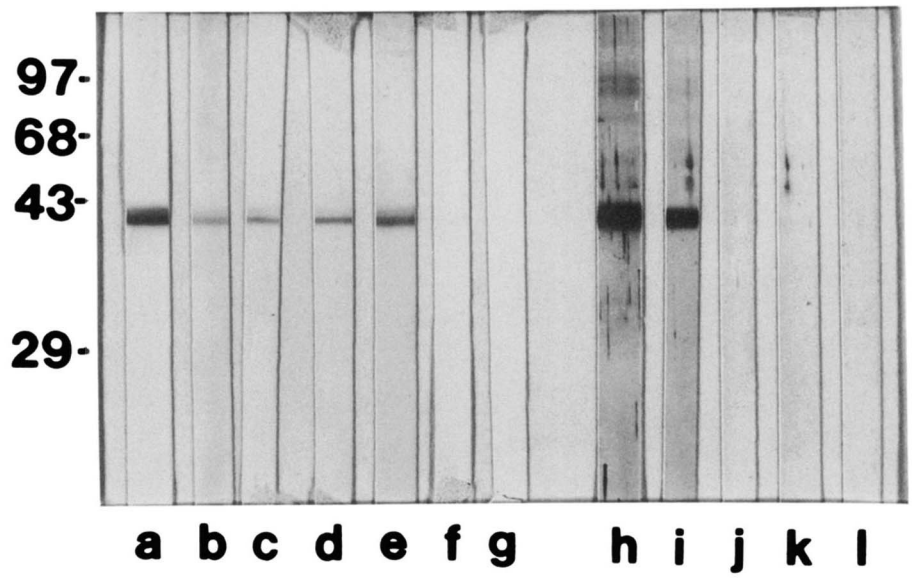

Figure 5. Immunoblot reactivity of purified La gp42 with patient sera, and analysis of lectin binding to purified La gp42. Blots were probed with sera pooled from patients infected with: *lane a, L. amazonensis* ($n = 4$); *lane b, L. major* ($n = 4$); *lane c, L. donovani* ($n = 3$); *lane d, L. chagasi* ($n = 4$); *lane e, T. cruzi* ($n = 4$). Normal human sera ($n = 4$) and no primary antibody controls are shown in *lanes f* and *g*, respectively. For lectin binding analyses, blots were probed with biotinylated lectins: *lane h*, Con A; *lane i*, wheat germ agglutinin; *lane j*, peanut agglutinin; *lane k*, PHA. The no biotinylated lectin control is shown in *lane 1*.

TABLE I

In vitro proliferation of PBMC from patients with cutaneous or mucosal Leishmaniasis to La gp42[a]

Patient	Mean cpm (SD) ($\times 10^{-3}$)						
	La lysate		La gp42		PHA		Medium
Cutaneous							
N.	15.3 (0.2)	11.0[b]	2.0 (0.4)	1.4[b]	ND		1.4 (0.1)
J.M.	40.3 (3.0)	51.0	35.3 (6.0)	44.0	ND		0.8 (0.3)
C.C.	114.4 (11.3)	47.0	7.2 (2.4)	3.0	84.9 (31.9)	35.0[b]	2.4 (1.5)
Mucosal							
D.J.	48.3 (12.3)	11.0	31.6 (11.3)	7.0	ND		4.6 (0.8)
A.B.	83.2 (23.0)	65.0	10.9 (3.2)	8.0	10.8 (1.1)	8.4	1.2 (0.5)
L.V.	31.3 (3.5)	182.0	12.8 (0.2)	131.0	ND		0.2 (0.1)
Normal[c]							
$n = 5$	ND		0.9 (1.2)		102.4 (55)		0.9 (1.3)

[a] PBMC were cultured for 5 days at 3 to 4 × 10^5 cells/well with *L. amazonensis* lysate (10 μg/ml); La gp42 (5 μg/ml); PHA (5 μg/ml) or medium. The wells were pulsed with 1 μCi of ^{3}H-thymidine for the final 18 h of culture.

[b] Stimulation index was calculated by dividing mean cpm of cells cultured with Ag by mean cpm of cells cultured without Ag.

[c] The results from five uninfected controls, tested individually, are expressed as one mean cpm (SD); range (0.1 to 3.0 × 10^3).

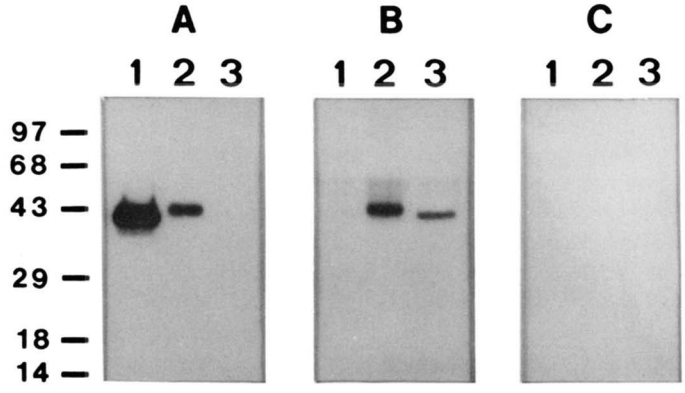

Figure 6. Immunoblot analysis of La gp42, Lm gp42, and Lc gp42 with rabbit antisera raised against La gp42 and Lc gp42. SDS-PAGE containing purified La gp42 (*lane 1*), Lm gp42 (*lane 2*), and Lc gp42 (*lane 3*) were transfered to nitrocellulose and probed with a rabbit anti-La gp42 serum (*A*), a rabbit anti-Lc gp42 serum (*B*), or normal rabbit serum (*C*). Bound antibody was detected with ^{125}I-protein A followed by autoradiography with exposure times of 4 h for *A* and 8 h for *B* and *C*.

the *L. amazonensis* Ag. No homology was observed between the N-terminal sequences of these two Ag and previously published protein sequences, including those of gp63. These data clearly indicate the homology between the 42-kDa GPI-anchored surface membrane proteins of *L. amazonensis* and *L. major*.

DISCUSSION

In this study, we have partially characterized an abundant 42-kDa surface antigen of *L. amazonensis*. Biochemically, we have shown that La gp42 is a glycosylated protein, expressed on the surface of promastigotes, and anchored to the parasite membrane via a PI-PLC cleavable GPI moiety. We were unable to detect La gp42 in amastigote Ag lystates by immunoblot analysis despite the demonstration of La gp42 mRNA in amastigotes by in vitro translation (data not shown). By immunoblot analyses and N-terminal sequence comparisons, we have provided definitive evidence demonstrating homology between La gp42 and a similar but less abundant promastigote Ag of *L. major*. La gp42 is strongly recognized serologically by patients infected with different species of *Leishmania*, as well as Chagas' disease patients. Additionally, La gp42 stimulated the in vitro proliferation of and IFN-γ secretion by PBMC from leishmaniasis patients.

Kahl, McMahon-Pratt, and co-workers (19, 20) identified a dominant 46-kDa promastigote-specific surface Ag (M-2) of *L. amazonensis* using a mAb that passively protected mice against infection with this parasite. This membrane glycoprotein was used successfully to immunize mice against cutaneous leishmaniasis (21). Compar-

Figure 7. N-terminal sequence homology between La gp42 and Lm gp42. The N-terminal sequences of La gp42 and Lm gp42 were determined from Ag separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The sequences and alignments are shown with identical residues (:) and mismatched residues (*) indicated. The symbol (– – –) represents unassigned residues of Lm gp42.

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L. major          Ala Gly Thr Ser Asp Phe Thr --- Ala Gln Gln Thr Asn Thr Leu Thr
                   :   :   :   :   :   :   :     :   :   :   :   :   :   :   :
L. amazonensis    Ala Gly Thr Ser Asp Phe Thr Glu Ala Gln Gln Thr Asn Thr Leu Thr

L. major          Val --- Gln Ala Phe Ala Arg Ala Ile --- Ala Val Gly Asp Thr ---
                   :       :   :   :   :   :   :   :     :   *   :   :   *
L. amazonensis    Val Leu Gln Ala Phe Ala Arg Ala Ile Pro Ala Leu Gly Asp Leu Leu
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ison of the N-terminal sequence of La gp42 with that of gp46/M-2 revealed the identity of these two molecules (22). The apparent discrepancy in m.w. is most likely due to differences in isolation protocols and reflects the loss of the GPI anchor during PI-PLC cleavage. The data obtained from the murine model system, combined with the results from our human studies, suggest that the immunologic recognition of La gp42 may be important in cutaneous leishmaniasis.

The surface location of La gp42 and its conservation between *L. amazonensis* and *L. major* indicate that it may be important in parasite growth and development. Two other surface Ag of *Leishmania* parasites studied extensively by a number of laboratories have been implicated in the ability of *Leishmania* to infect macrophages. The first is gp63, a glycosylated, GPI-anchored surface Ag of pathogenic *Leishmania* species, which in addition to a potential receptor function, possesses an acid protease activity (23–29). The second is a unique glycoconjugate, LPG, also believed to be necessary for the intracellular survival of the parasite within macrophages (30–32). Of additional interest is the fact that host immune responses to these surface Ag appear important and as with La gp42, both gp63 and LPG have been shown to have potential as protective Ag in murine studies (33, 34). Serologically, native gp63 was also recognized by most leishmaniasis patient groups (12, 23, 35). The evaluation of patient cellular immune responses to both native and recombinant gp63 from various *Leishmania* parasites indicates that this Ag, like La gp42, induces strong T cell proliferative responses as well as production of IFN-γ in vitro.[4] As yet, little is known concerning the immunologic recognition of LPG during human disease.

The present report focuses on *L. amazonensis*, a causative agent of human cutaneous leishmaniasis. Most previous studies on the serologic recognition of defined *Leishmania* Ag focused mainly on the agents of visceral disease. Using human infection sera, Reed et al. (12) identified several cross-reactive antigens of *Leishmania*, as well as Ag specific to *L. chagasi*. In other studies, two *L. donovani* membrane proteins recognized by visceral leishmaniasis patient sera were identified, purified, and characterized biochemically (36, 37). One of these Ag, dp72, was also used to successfully immunize mice against experimental visceral leishmaniasis (38). Considering the broad recognition of La gp42 by various leishmaniasis and Chagas' disease patient sera, it could be of interest to identify and characterize the Ag of other *Leishmania* species that may be homologous to La gp42.

In light of our present understanding of protective immunity against leishmaniasis, the ability of La gp42 to stimulate T cells from leishmaniasis patients is significant. It is clear that recovery from disease and resistance to reinfection are dependent on the development of cell-mediated immune responses. For example, the stimulation of the Th1 subset of T cells in mice and the production of IFN-γ and IL-2 are necessary for the resolution of infection (4–6). In human leishmaniasis, protective immunity correlates with the development of a positive skin test and the ability of patient T cells to respond in lymphocyte proliferation assays in an Ag-specific manner (1, 2, 13, 39). As in the murine studies, selected cytokine production also appears essential considering the success of clinical trials involving the treatment of human visceral disease with antimony and IFN-γ (40).

From our studies, it appears that La gp42 may be one of the Ag important in the induction of protective responses. However, the complexity in the T cell responses of leishmaniasis patients to defined parasite Ag is great. Melby et al. (7, 8) showed that leishmaniasis patient T cells proliferated and secreted IFN-γ upon recognition of as many as 50 to 70 distinct parasite Ag. In these studies the responses varied among individual patients with disease state being a contributing factor. Other studies by Reed et al. (9) identified two glycoproteins of *L. chagasi*, Lc gp42 and Lc gp30, which were effective in stimulating T cells from a variety of leishmaniasis patient groups. Both Ag consistently elicited high proliferative responses as well as IL-2 and IFN-γ production by T cells from patients recovered from visceral or cutaneous leishmaniasis or with chronic Chagas' disease. Using monospecific rabbit antisera we have shown that La gp42 appears to be unrelated to the previously described 42-kDa glycoprotein of *L. chagasi*. Although some serologic cross-reactivity was noted between the *L. major* and *L. chagasi* Ag, we have obtained a partial N-terminal sequence for Lc gp42 that shows no homology to those of La gp42 and Lm gp42. Nevertheless, we demonstrated that La gp42, like Lc gp42, elicited T lymphocyte proliferation and IFN-γ production. Similar to the studies of Melby et al. (7, 8), however, variability between the responses of individual patients was apparent. The significance of such variability on the outcome of *Leishmania* infections remains to be determined.

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