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Mimetic Membrane System to Carry Multiple Antigenic Proteins from *Leishmania amazonensis*

Fabiana R. Santos[1], Denise B. Ferraz[1], Katia R.P. Daghastanli[2], F.Juarez Ramalho-Pinto[1], Pietro Ciancaglini[2]
[1]Departamento de Bioquímica e Imunologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, 14049-900 Ribeirão Preto, São Paulo, Brasil
[2]Departamento de Química, Faculdade de Filosofia Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, 14040-901 São Paulo, Brasil

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Abstract. Liposomes have long been used as models for lipid membranes and for the reconstitution of a single or multiple proteins. Also, liposomes have adjuvant activity in vaccines against several protozoan or bacterial organisms. Thus, the main objective of the present study was to obtain a crude extract of detergent-solubilized proteins of *Leishmania amazonensis* amastigotes and reconstitute them into liposomes. Neutral and zwiterionic detergents were less efficient than an ionic detergent. In order to obtain efficient solubilization using only sodium dodecyl sulfate (SDS), the effects of detergent and protein concentration and incubation time were studied. The maximum of solubilized proteins was obtained instantaneously using a ratio of 0.5 mg/ml of protein to 0.1% (w/v) detergent at 4°C. Dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylserine (DPPS) and cholesterol in a weight ratio of 5:1:4 were used for protein reconstitution into liposomes using the cosolubilization method, yielding 60% of incorporation. The incorporation of multiple parasite proteins results in a vesicular diameter of proteoliposomes of about 140 nm, presenting a final lipid weight ratio for DPPC, DPPS and cholesterol of 1:1:5, with high stability. The detergent-solubilized proteins of *L. amazonensis* amastigotes present in the proteoliposome, when analyzed by SDS-polyacrylamide gel electrophoresis, include a wide range of parasite-incorporated proteins. BALB/c mice inoculated with these proteoliposomes were able to produce antibodies against the proteins reconstituted in DPPC:DPPS:cholesterol liposomes and were partially resistant to infection with *L. amazonensis* promastigotes. These results indicate that this system can be used as a possible vaccine against *L. amazonensis*.

Key words: *Leishmania amazonensis* — Membrane protein — Detergent solubilization — Liposome — Proteoliposome — Cosolubilization method

Correspondence to: P. Ciancaglini; email: pietro@ffclrp.usp.br

Introduction

While lipids are the fundamental structural elements of cell membranes, proteins are responsible for carrying out specific membrane functions. When a foreign organism invades a mammalian cell, these molecules are important in the first contact with the host cell during the invasion of parasites (Mitchell et al., 2004; Hespanhol et al., 2005). To bypass the use of intact dead parasites to induce protective immunity into experimental animals, integral membrane proteins reconstituted in liposomes have been used (Lezama-Davila, 1997). Because the techniques used to recover membrane proteins may also disorganize the native protein structure, a challenge in many membrane studies is to ensure that the protein function is not altered or lost when removed from the membrane by solubilization and purification procedures (le Maire, Champeil & Moller, 2000). One approach to avoid membrane protein denaturation is to reincorporate these molecules back into a mimetic membrane structure, a process generally referred to as "reconstitution" (Rigaud, 2002). The reconstitution of membrane proteins into liposomes is a useful tool to prepare antigenic components to induce immunity in experimental models (de Jonge et al., 2004; Kita et al., 2005). In addition to preserving the active structure of proteins, liposomes have adjuvant

activity in vaccines against protozoan parasites (Afrin et al., 2002; Mazumdar, Anam & Ali, 2004; Uemura et al., 2005).

During their life cycle, the protozoan parasites of the genus *Leishmania* replicate and differentiate in the gut of the sandfly as flagellated promastigotes and are transmitted to a vertebrate by the vector. Survival of this parasite within the vertebrate host is dependent on successful entry into a macrophage and transformation of promastigotes into the amastigote form (Courret et al., 2001). During these processes, the surface of the parasite undergoes considerable changes to adapt alternatively to the different environments within the vertebrate and invertebrate hosts.

In the present study, we prepared a crude extract of detergent-solubilized proteins of *Leishmania amazonensis* amastigotes and reconstituted them into liposomes, employing the cosolubilization method (Seddon, Curnow & Booth, 2004; Daghastanli et al., 2004). The experiments were designed to study the effects of detergent and protein concentration, time and incubation temperature on *L. amazonensi*s protein solubilization, followed by a standard procedure of proteoliposome construction. We further employed this material to induce an immune response in BALB/c mice and were able to show both an antibody response and significant protection from homologous infection. These results with proteoliposomes indicate a possible application of these mimetic membrane systems in the preparation of a putative vaccine against promastigotes of *L. amazonensi*s.

Materials and Methods

Materials

All solutions were prepared using Millipore (Bedford, MA) DirectQ ultrapure apyrogenic water. Tris(hydroxymethyl)aminomethane (TRIS), phosphate-buffered saline (PBS), bovine serum albumin (BSA), Schneider' insect medium, molecular weight markers (10–205 kDa), ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), 1,10-phenanthroline, dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylserine (DPPS), cholesterol, sodium dodecyl sulfate (SDS), 3-([3-cholamidopropyl]dimethyl-ammonio)-1-propane sulfonate (CHAPS), 3-([3-cholamidopropyl]dimethyl-ammonio)-2-hydroxy-1-propane sulfonate (CHAPSO), polyoxyethylene 9-lauryl ether (Polidocanol), polyoxyethylene-sorbitan monolaurate (Tween 20) and 3,3′-diaminobenzidine (DAB) were purchased from Sigma (St. Louis, MO); peroxidase-labeled goat anti-mouse immunoglobulin G (IgG) conjugate was from Southern Biotechnology (Birmingham, AL); Calbiosorb resin was from Calbiochem (San Diego, CA).

Mice and Parasites

Female BALB/c mice, 8–12 weeks old, were bred and maintained under standard conditions in the animal facility at the Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo. The parasite strain IFLA/BR/67/PH8 of *L. amazonensis* was maintained in BALB/c mice (Noronha et al., 1998; Nunes et al., 1997). The amastigotes were isolated from footpad lesions of infected mice and transferred to Schneider's medium. The parasite suspension was centrifuged at $1,200 \times g$ for 10 min, and the pellets were collected and stored at −20°C.

Preparation of Crude Extract of *L. Amazonensis*

The frozen pellets of amastigotes were resuspended in 5 mM TRIS-HCl buffer (pH 7.5), containing 1 mM EDTA, 1.6 mM PMSF, 0.1 mM E-64 and 1 mM 1–10 phenanthroline. The suspension was sonicated at 4°C with three 30-s blasts at 60 W.

Estimation of Protein Concentration

Protein concentration was estimated in the presence of SDS 2.0% (w/v) by the procedure described by Hartree (1972), using crystallized BSA as standard. To estimate protein concentration in the fractions collected after isopycnic density gradient centrifugation, the method described by Read & Northcote (1981) was employed, also using crystallized BSA as standard.

Antiserum Preparation

BALB/c mice were infected subcutaneously in the hind footpad with 10^6 stationary growth phase *L. amazonensis* promastigotes. Two months after infection, the animals were bled and the antiserum against total *L. amazonensis* antigenic determinants was collected.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting

The determination of the molecular weight of proteins was achieved by SDS-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (1970). Briefly, 5% polyacrylamide was used for the stacking gel and 10% for the running gel (1.0 mm thick). The gels were silver-stained to visualize the profile of the protein bands. Molecular weight markers ranged 10–205 kDa. Western blotting was performed by electrophoretically transferring the protein bands to a nitrocellulose membrane. The membrane was treated with blocking buffer (PBS, containing 5% [w/v] nonfat milk and 0.05% [v/v] Tween 20) for 1 h and probed with antiserum against total *L. amazonensis* at a dilution of 1:75 in the blocking buffer for 1 h. Excess proteins were removed with four washes of 5 min with washing buffer (PBS, containing 0.05% [v/v] Tween 20). Finally, the membrane was probed with peroxidase-labeled goat anti-mouse IgG conjugate at a 1:1,000 dilution in the washing buffer. The DAB substrate was used to reveal protein-antibody interactions at the concentration of 0.5 mg/ml.

Solubilization of *L. Amazonensis* Membrane Proteins

Samples of crude extract of parasite (0.5 mg/ml of protein) were first incubated for 2 h at 25°C with different detergents in the concentration range of about 0.1–7% (w/v). Solubilized proteins were then separated by ultracentrifugation at $100,000 \times g$ for 1 h. Optimal solubilization condition was determined by changing protein or detergent concentration and incubation time of the protein sample with the detergent. The solubilized protein

concentration was estimated in the supernatant and in the resuspended pellets to calculate the yield of solubilization.

Proteoliposome Preparation

Proteoliposomes were prepared with DPPC, DPPS and cholesterol at different weight ratios. The lipid mixture was dissolved in 1 ml chloroform and dried under nitrogen flow, and the lipid film formed was maintained under vacuum for 1 h. Then, 2 ml of a solution of 5 mM TRIS-HCl (pH 7.5) containing 25 mg/ml SDS was added to the film. The mixture was incubated at 60°C for 1 h and vortexed at 10-min intervals. After that, solubilized lipids were sonicated, using the microtip, for 2 min at 240 W. One milliliter of SDS-solubilized protein extract (0.5 mg/ml) was added to this mixture, which was then incubated for 45 min at room temperature. The detergent was removed from the mixture using 200 mg/ml Calbiosorb hydrophobic resin. After detergent removal, the mixture was centrifuged at 140,000 × *g* for 1 h and the pellet, constituted by proteoliposomes, was resuspended in 1 ml of 5 mM TRIS-HCl. Liposomal formulations in the absence of protein were prepared with identical lipid composition and used as control.

Estimation of Lipid Concentration

The lipid constitution of proteoliposomes or liposomes was determined by thin-layer chromatography (TLC, silica plate) after extraction with chloroform:methanol (1:1 v/v) using as primary solvent chloroform:methanol:ammonia (65:35:5 v/v) and as secondary solvent chloroform:methanol:acetone:acetic acid:water (10:2:4:2:1 v/v) in the same dimension. After lipid detection, using iodine vapor, the silica spots were scraped and treated with chloroform and the phospholipids and cholesterol were quantified as described by Chen, Toribara & Warner (1956) and Higgins (1987), respectively.

Isopycnic Density Gradient Centrifugation

A continuous sucrose gradient was prepared as described by Daghastanli et al. (2004) (1–30% sucrose in 5 mM TRIS-HCl [pH 7.5], density ranging 1.0381–1.1764) using a Gradient Maker (Hoeffer Scientific Instruments, San Francisco, CA). Samples of SDS-solubilized protein extract, liposomes or proteoliposomes (500 μl) were loaded on the gradient and centrifuged at 180,000 × *g* for 4 h at 25°C. The gradients were then fractioned into 500 μl fractions and analyzed for protein and inorganic phosphate content (Chen et al., 1956).

Light Scattering of Proteoliposomes

The liposome and/or proteoliposome size distribution was determined by dynamic light scattering using a Zetasizer 3000HS_A (Malvern Instruments, Malvern, UK). Proteoliposomes were stored at 4°C, and the diameter average was monitored for 15 days.

Evaluation of Protection against Infection with *L. AMAZONENSIS*

Groups of five animals were immunized intraperitoneally (i.p.) with liposome, 20 μg of SDS-solubilized protein extract or proteoliposome (10, 20 or 40 μg). The control group received PBS. At 3 weeks after immunization, mice were challenged with one subcutaneous injection in the right hind footpad with 10^6 stationary growth phase *L. amazonensis* promastigotes. At various times after infection, footpads were measured with a caliper (125MEA-6/150; Starrett, São Paulo Brazil). Lesion size was determined by subtracting the caliper-measured thickness of the uninfected contralateral footpad from that of the infected one.

Results

Solubilization of *L. AMAZONENSIS* Membrane and Identification of Antigenic Proteins

A typical preparation of crude extract of *L. amazonensis* by sonication yields around 44% of water-soluble proteins. By Western blotting, these proteins were shown to be reactive to an antiserum generated by inoculation of live promastigotes into mice (*data not shown*). To enhance the process of solubilization, frozen pellets of amastigotes were resuspended in TRIS buffer and sonicated, protein was quantified and detergent was added.

The capacity of the detergent to solubilize the crude extract was studied using different classes of surfactants in the concentration range of about 0.05–7.0% (w/v) and using different times and temperatures of incubation and different protein concentrations. The soluble protein recovery of crude extract of *L. amazonensis* (0.5 mg/ml) treated for 2 h at 25°C with 0.4% (w/v) CHAPS, 0.3% (w/v) CHAPSO, 1% (w/v) Tween 20 or 5% (w/v) Polidocanol were similar, yielding approximately 59% of the parasite proteins. Our results indicate that the most efficient detergent at solubilizing *L. amazonensis* membrane proteins was SDS at a concentration of 0.1% (w/v), giving a recovery of 93%.

To visualize the proteins present in the crude extract of *L. amazonensis* solubilized with the various detergents, a silver stain method was used. Our results, presented in Figure 1A, indicate that almost every preparation displayed a similar pattern of protein recovery. However, the SDS-solubilized protein extract, as expected from protein determination, gave higher yields (Fig. 1A, lane 5a). Consistent with this finding, the unsolubilized pellet displayed only discrete protein bands (Fig. 1A, lane 5b). The electrophoretic profile of supernatants and pellets was very similar when the solubilization of *L. amazonensis* membrane proteins was achieved with the other detergents (Fig. 1A, lanes 2, 3, 4, 6, a and b). Figure 1B shows the antigenic profiles of crude extract and SDS-solubilized protein extract after reaction with antiserum against total *L. amazonensis* antigenic determinants (lanes 8 and 9, respectively).

In order to determine the optimal conditions for efficient solubilization of proteins with SDS from the crude extract of *L. amazonensis*, the effects of this detergent dependent on protein concentration, incubation time and temperature were studied. Figure 2 shows the amount of protein solubilized as a function of SDS concentration for a fixed amount of protein in

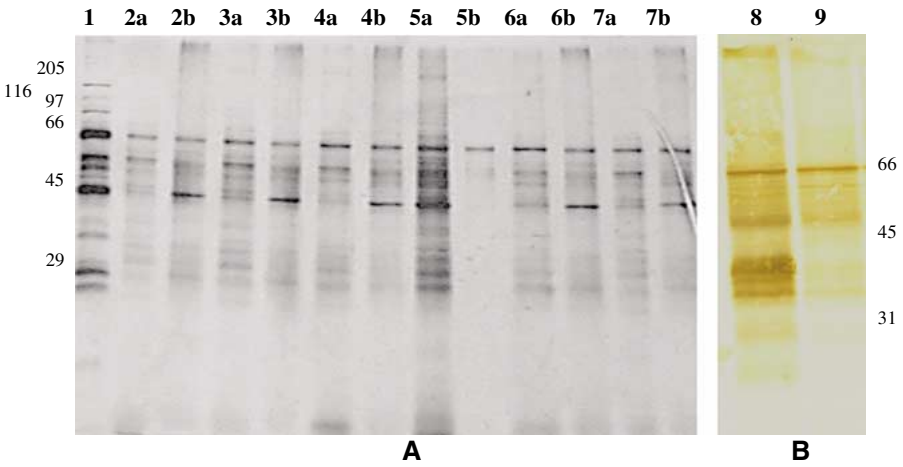

Fig. 1. (*A*) Silver-stained SDS-PAGE gel of proteins from *L. amazonensis*. Aliquots of 0.5 mg/ml of crude extract were mixed at 25°C with different detergents, as described in Materials and Methods. Supernatants containing solubilized proteins are shown in lanes labeled *a* and resuspended pellet in lanes labeled *b*. Lanes: *1*, molecular mass standard proteins in kDa; *2*, 0.4% (w/v) CHAPS; *3*, 0.3% (w/v) CHAPSO; *4*, 1% (w/v) Tween 20; *5*, 0.1% (w/v) SDS; *6*, 5% (w/v) Polidocanol and *7*, without detergent (control). (*B*) Western blotting analysis of (*lane 8*) crude extract and (*lane 9*) SDS-solubilized protein extract with antiserum prepared as described in Materials and Methods.

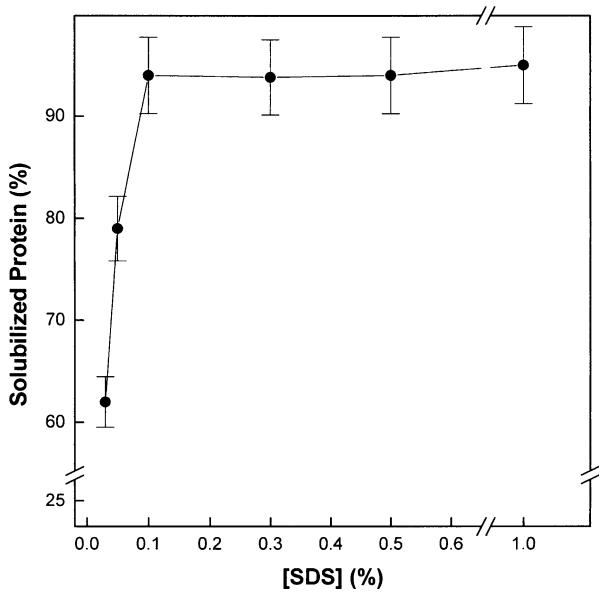

Fig. 2. Effect of SDS concentration on the solubilization of crude extract of *L. amazonensis*. Aliquots of 0.5 mg/ml of crude extract were mixed at 25°C with SDS at different concentrations. After 2 h, the solubilized proteins were separated by centrifugation at 100,000 × *g* for 1 h.

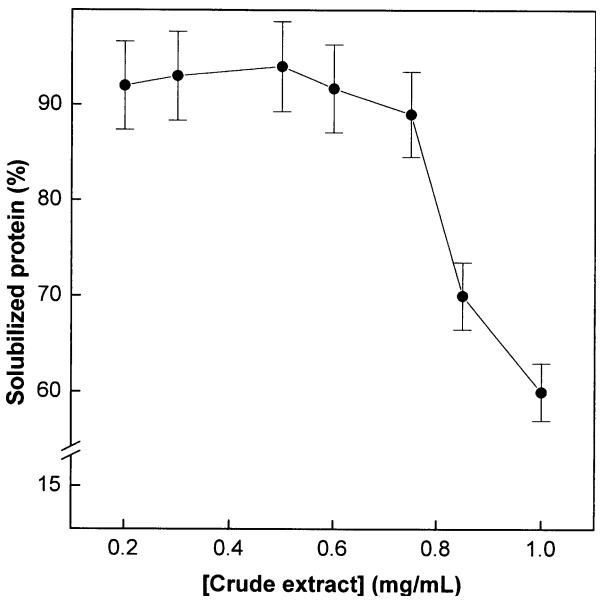

Fig. 3. Effect of protein concentration on the solubilization of crude extract of *L. amazonensis*. Different concentrations of crude extract were mixed at 25°C with 0.1% (w/v) SDS. After 2 h, the solubilized proteins were separated by centrifugation at 100,000 × *g* for 1 h.

the extract. Under these conditions, the amount of solubilized proteins increased steadily, reaching a maximum at a detergent concentration of 0.1% (w/v). Higher concentrations of SDS did not increase the amount of proteins recovered in solution. Changes in protein concentration for the fixed SDS concentration of 0.1 % (w/v) showed that 0.5 mg/ml of crude extract gave the maximum yield of 94% (Fig. 3). The degree of solubilization decreased as protein concentration increased above 0.5 mg/ml, suggesting that this amount of detergent was insufficient to promote solubilization of the larger quantities of membrane proteins. Accordingly, using a ratio of 0.5 mg/ml of protein to 0.1% (w/v) SDS at 4°C, the solubilization of crude extract was achieved almost instantaneously (*data not shown*).

It is important to remark that the SDS-solubilized protein extract of *L. amazonensis* contains little

phospholipid and sterol, resulting in 27 and 38 μg/mg of total protein, respectively, when determined after chloroform extraction.

Proteoliposome Preparation and Characterization

In order to prepare proteoliposomes, different mixtures of lipids (DPPC or DPPS alone or both phospholipids with or without cholesterol, in different proportions) and SDS-solubilized protein extract were used and optimization of protein incorporation was obtained using the mixtures of DPPC, DPPS and cholesterol at weight ratios of 5:1:4 and 0.5 mg/ml of protein. The incorporation of proteins into liposomes by the cosolubilization method requires complete removal of the detergent. Here, SDS removal was achieved by batch hydrophobic adsorption with the Calbiosorb resin, giving an SDS-free proteoliposome. In our system, the reconstitution of solubilized proteins was around 60%.

To determine the stability of the proteoliposomes in TRIS buffer, a dynamic light scattering assay was used. This assay revealed that the average diameter of the proteoliposome was 140 nm soon after preparation and 150 nm after 15 days, indicating a high stability of this system.

Data presented in Table 1 indicate that lipid composition of liposome results in considerable loss of amount of DPPC and cholesterol, exhibiting a marked change of the initial lipid proportion present in solution. Also, during the formation of the proteoliposome a high loss of both phospholipids occurs compared with the concentration of remaining cholesterol. Probably, this process occurs to incorporate and stabilize the proteins in the vesicular system besides the loss by resin adsorption and/or the formation of liposomes free of protein.

To characterize the degree of incorporation of *L. amazonensis* proteins into proteoliposomes, an isopycnic density gradient centrifugation was performed, and the fractions were assayed for protein and phosphate contents. As shown in Figure 4A, analysis of SDS-solubilized protein extract revealed the presence of proteins in the middle region of the gradient (around 14.2–16.2% sucrose concentration, density 1.058–1.064 g/cm^3). With liposomes, a peak corresponding to the inorganic phosphate content occurred at 12% sucrose, density 1.046 g/cm^3 (Fig. 4B). Finally, proteoliposomes showed a single peak at 11.3% sucrose concentration (1.042 g/cm^3), for both the inorganic phosphate and protein, indicating efficient incorporation during reconstitution of SDS-solubilized protein extract (Fig. 4C).

SDS-PAGE of proteoliposomes revealed a variety of proteins with a distribution similar to that observed in the SDS-solubilized protein extract of *L. amazonensis*, indicating nonselective incorporation of parasite proteins into liposomes (Fig. 5A, lane 3).

Table 1. Lipid composition (in w/w and mol/mol proportion) of the liposome and proteoliposome determined by silica plate TLC as described in Materials and Methods

Sample	DPPC:DPPS: cholesterol (w/w) ratio	DPPC:DPPS: cholesterol (moles/moles) ratio
Initial mixture	5:1:4	5:1:7
Liposome	3:1:3	3:1:6
Proteoliposome	1:1:5	1:1:9

To determine whether BALB/c mice inoculated with proteoliposome were capable of producing antibodies against *L. amazonensis* proteins, five animals were inoculated with one i.p. injection of 20 μg of the material and, 3 weeks later, bled and their sera pooled. Our results of immunoblotting with SDS-solubilized protein extract, which had been electrophoretically separated by SDS-PAGE and probed with serum from mice inoculated with proteoliposome, indicate that these animals were able to produce antibodies against *L. amazonensis* proteins present in the SDS-solubilized protein extract reconstituted into DPPC:DPPS:cholesterol liposomes (Fig. 5B, lane 5).

To evaluate whether the proteoliposomes-carrying *L. amazonensis* antigens could induce an immune response in BALB/c mice to generate some degree of protective immunity to a homologous infection, mice were inoculated with different concentrations of the preparation (Table 2). Our results indicate that mice injected with proteoliposomes that contained parasite antigens were able to counteract the promastigote infection in a dose-response manner. Mice inoculated with 10–40 μg of proteoliposome presented a significant reduction in the size of the footpad lesion at 12 weeks of infection, with less skin damage at higher concentration of antigen (Table 2).

Discussion

Liposomes have been used in experimental therapeutics as carriers for drugs (Pinto-Alphandary, Andremont & Couvreur, 2000) or as immunoadjuvants for vaccine preparations (Gregoriadis, 1990). To achieve these goals, proteoliposome systems can be prepared by a variety of techniques, such as mechanical dispersion, sonication, extrusion, solvent dispersion, reverse phase evaporation and cosolubilization with detergents (Camolezi et al., 2002). As a preliminary stage to investigate the possibility of using liposomes to induce protective immunity in mice to homologous infection, we incorporated SDS-solubilized protein extract of *L. amazonensis* into negatively charged liposomes.

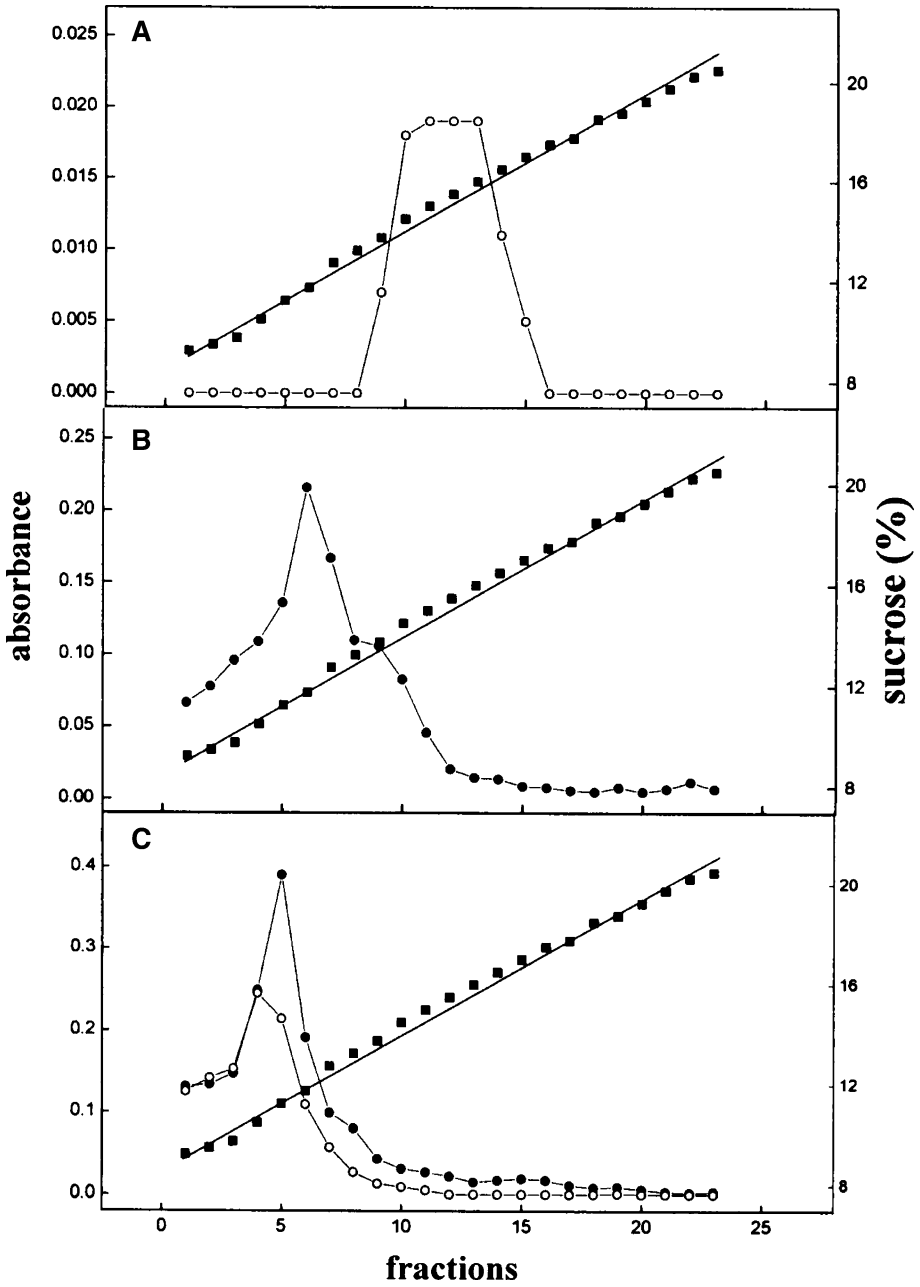

Fig. 4. Isopycnic density sucrose gradient centrifugation study. (*A*) SDS-solubilized protein extract of *L. amazonesis*. (*B*) Liposome. (*C*) Proteoliposome. ○, Protein, absorbance at 595 nm; ●, lipid, absorbance at 820 nm; and ■, percent sucrose.

Negatively charged liposomes have been shown to be efficient vehicles for specific delivery of anti-*Leishmania* drugs to mouse macrophages (Tempone et al., 2004). Accordingly, we prepared proteoliposomes with DPPC, DPPS and cholesterol, as a way to present *Leishmania* antigens to scavenger receptors, which are present in macrophages and dendritic cells (Harshyne et al., 2003; Shakushiro et al., 2004; Greaves & Gordon, 2005).

In view of the important role played by the cell-surface molecules of pathogenic protozoa in their interaction with the host, some effort has been devoted to the characterization of these membrane components. Because the components of *L. amazonensis* membrane appear to be associated with its pathogenic activity, it has been suggested that generating an immune response against these components could lead to the development of immunoprophylaxis against cutaneous leishmaniasis (Sacks & Noben-Trauth, 2002). Two major surface molecules, lipophosphoglycan and the zinc-metalloproteinase gp63, have been studied (Descoteaux & Turco, 1999; Ilgoutz & McConville, 2001).

We chose amastigotes of *L. amazonensis* as the source for antigen because developmentally regulated molecules may be biologically important for the intracellular survival of the parasite. Furthermore, the amastigote is the stage of *Leishmania* parasites

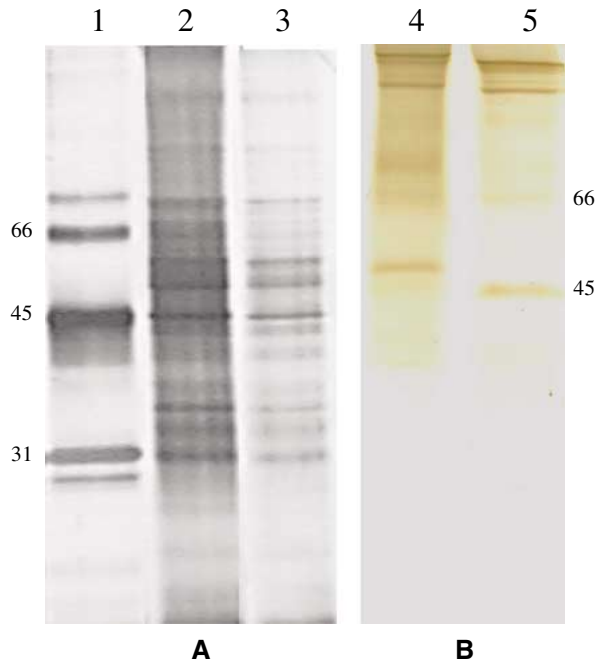

Fig. 5. (*A*) Silver-stained SDS-PAGE gel of proteins from *L. amazonensis*. *Lane 1*, molecular mass standard proteins in kilodaltons; *lane 2*, SDS-solubilized protein extract; and *lane 3*, proteins present in proteoliposomes. (*B*) Western blotting analysis of SDS-solubilized protein extract with: *lane 4* antiserum prepared as described in Materials and Methods and (*lane 5*) sera collected 3 weeks after immunization intraperitoneal from mice with 20 μg of proteoliposome.

responsible for the pathology associated with this disease, and antigens from the amastigote have been previously used in the construction of a leishmanial vaccine by others (Rafati et al., 2000; Aebischer et al., 2000). Furthermore, the efficacy of immunization of BALB/c mice with purified soluble amastigote proteins has been reported for three amastigote antigens (P-2, P-4 and P-8), which provided significant protection against infection with *L. pifanoi* (Soong et al., 1995).

A successful strategy for membrane reconstitution involves three main aspects: (1) partial or complete solubilization of the membrane protein and lipid components, (2) preservation of the biological activity of the solubilized proteins and (3) mixing of the solubilized purified protein and lipids in order to achieve reconstitution (Silvius, 1992). To be amenable to being incorporated into liposomes, the membrane protein components must first be solubilized. Many detergents have been used to obtain solubilized proteins from membranes, namely sodium deoxicholate, SDS, sodium cholate, Empingem BB and octylglucopyranoside (Parmar, Edwards & Madden, 1999; Daghastanli et al., 2004). The process of membrane protein solubilization is affected by both the ionic character and the critical micelar concentration of the detergent (Santos & Ciancaglini, 2000). The solubilization of membrane proteins of *L. amazonensis* with neutral and zwiterionic detergents was shown to be less efficient than with the anionic detergent SDS (Fig. 1A, lane 5a). Although SDS is known as a denaturing detergent, in our hands it showed higher solubilization capacity (94%) without a significant loss of antibody-binding ability of the solubilized material (Fig. 1B, lane 9).

Table 2. Level of protection against *L. amazonensis* promastigotes of BALB/c mice immunized with different concentrations of proteoliposome

Immunized Group[a]	Lesion size (mm)[b]	(%) Reduction
PBS	7.9 ± 1.1	–
Liposome	6.5 ± 0.7	18
SDS-solubilized protein extract (20 μg)	7.9 ± 0.7	0
Proteoliposome (10 μg)	5.9 ± 0.5	25
Proteoliposome (20 μg)	5.1 ± 0.2	35
Proteoliposome (40 μg)	4.1 ± 0.5	48

[a] BALB/c mice were immunized i.p. with liposome, SDS-solubilized protein extract or proteoliposome as described in Materials and Methods. Mice given PBS were used as control. At 3 weeks after immunization, all animals were challenged with 10^6 *L. amazonensis* promastigotes.
[b] Lesion size represents the mean lesion diameter in mm ± SE of lesions developing in groups of five animals at 12 weeks postinfection.

Antigenic proteins present in the SDS-solubilized protein extract were used to prepare proteoliposomes by a cosolubilization method in which detergent, lipid and protein were mixed. A critical factor in this technique is complete removal of the detergent (Rigaud et al., 1998, Daghastanli et al., 2004). Among the usual methods for detergent removal, the hydrophobic resin is preferable because it is very rapid and does not dilute the sample (Haaker & Racker, 1979; Furth et al., 1984; Jones et al., 1988; Silvius, 1992; Rigaud et al., 1998). Using the Calbiosorb resin, as described by Daghastanli et al. (2004), SDS removal can be as high as 99%. In our hands, the use of Calbiosorb resin for detergent removal allowed 60% incorporation of SDS-solubilized protein extract into proteoliposomes.

Our results with sucrose density gradients to characterize the protein incorporation in the lipid bilayer of lamellar liposomes indicate that liposomes reconstituted with DPPC, DPPS and cholesterol present a wide peak of phosphate originated from acid hydrolysis of phospholipids, indicating the presence of different liposome populations distributed in two close density ranges (Fig. 4B). Proteoliposomes showed a single symmetric elution profile, as detected by phosphate and protein estimation, indicating a remarkably homogeneous distribution of

the vesicle population and the consistent absence of other lipid-protein aggregates (Fig. 4C). Furthermore, these systems appear in lower densities probably because of their cholesterol content, as shown in Table 1 and described by other authors (Cevc & March, 1987). Our results clearly indicate that the method employed here may lead to efficient protein reconstitution, reaching a yield of 60% in a homogeneous proteoliposome population having a similar density to that of the single lipid vesicle.

Incorporation of the protein into vesicles depends on many factors but mainly on the length of the fatty acid hydrocarbon chains and the type of polar head group of phospholipid component in the liposomes, as recently shown for antigenic membrane proteins of *Pasteurella multocida* (Daghastanli et al., 2004). Our results indicate that *L. amazonensis* membrane proteins are efficiently incorporated in proteoliposomes containing palmitoyl acyl chain, present also in phosphatidylcholine and phosphatidylserine phospholipids.

The liposome bilayer composition has an important effect on its interaction with tissues and might be critical for targeting to specific antigen-processing cells. Anionic liposomes containing phosphatidylcholine, cholesterol and phosphatidylglycerol or phosphatidylserine interact with a small percentage of human and murine dendritic cells (Foged et al., 2004). In contrast, inclusion of mannosylated phosphatidylethanolamine in the design of cationic liposomes, intended for targeting the mannose receptor, increases the interaction of these vesicles with dendritic cells. Also, cationic liposomes interact with a very high percentage of both human and murine dendritic cells (Foged et al., 2004).

Membrane antigens of *L. donovani* promastigotes, when entrapped in liposomes, can induce very significant levels of protection against infection in both hamsters and BALB/c mice (Afrin & Ali, 1997; Ali & Afrin, 1997; Afrin, Anam & Ali, 2000). In mice, the extent of protection conferred by these antigens varies depending on the overall surface charge of the proteoliposome. It has been shown that liposomes containing phosphatidylserine entrapped with pentavalent antimony are taken up by macrophages via scavenger receptors, these systems being 16-fold more effective against *L. chagasi*-infected macrophages than the free drug (Tempone et al., 2004).

This report presents the biophysical characteristics of the proteoliposomes and a preliminary study of humoral immunological reactivity and protective immunity of BALB/c mice immunized with these proteoliposomes. We have shown that a careful choice of the mixture DPPC, DPPS and cholesterol used to obtain proteoliposomes was essential to optimize protein incorporation. Furthermore, these studies resulted in a proteoliposome constituted by multiple *L. amazonensis* proteins, which not only were recognized by the antiserum against total *L. amazonensis* antigenic determinants but also generated considerable level of protective immunity in mice (Table 2). Accordingly, we are confident in suggesting that this proteoliposome system could be used as a carrier for antigenic proteins from *L. amazonensis*, thus contributing to the development of potential vaccine candidates for cutaneous leishmaniasis prevention.

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