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RESEARCH ARTICLE

Nanobiotechnologic approach to a promising vaccine prototype for immunisation against leishmaniasis: a fast and effective method to incorporate GPI-anchored proteins of *Leishmania amazonensis* into liposomes

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Abstract

Liposomes are known to be a potent adjuvant for a wide range of antigens, as well as appropriate antigen carriers for antibody generation response *in vivo*. In addition, liposomes are effective vehicles for peptides and proteins, thus enhancing their immunogenicity. Considering these properties of liposomes and the antigenicity of the *Leishmania* membrane proteins, we evaluated if liposomes carrying glycosylphosphatidylinositol (GPI)-anchored proteins of *Leishmania amazonensis* promastigotes could induce protective immunity in BALB/c mice. To assay protective immunity, BALB/c mice were intraperitoneally injected with liposomes, GPI-protein extract (EPS_{GPI}) as well as with the proteoliposomes carrying GPI-proteins. Mice inoculated with EPS_{GPI} and total protein present in constitutive proteoliposomes displayed a post-infection protection of about 70% and 90%, respectively. The liposomes are able to work as adjuvant in the EPS_{GPI} protection. These systems seem to be a promising vaccine prototype for immunisation against leishmaniasis.

Keywords

Antigenic membrane protein, *Leishmania amazonensis*, nanoliposomes, protective immunity, vaccine prototype

History

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Introduction

The leishmaniasis are prevalent in 98 countries and 3 territories on 5 continents. Approximately 1.3 million new cases occur annually, of which 300 000 are visceral (90% of which occur in Bangladesh, Brazil, Ethiopia, India, Nepal, South Sudan and Sudan) and 1 million are cutaneous (occurring mainly in Afghanistan, Algeria, Brazil, Colombia, the Islamic Republic of Iran, Pakistan, Peru, Saudi Arabia, the Syrian Arab Republic and Tunisia) or mucocutaneous (mainly occurring in Brazil, Peru and the Plurinational State of Bolivia). Of the 1.3 million estimated cases, only less than a half are actually reported (Alvar et al., 2012; WHO, 2013).

Leishmania amazonensis is a species of great medical interest because it is responsible for at least two clinical forms of leishmaniasis, a localised cutaneous leishmaniasis (LCL), characterised by benign single lesions, and diffuse cutaneous leishmaniasis (DCL), a rare form of cutaneous leishmaniasis that typically begins as localised papules or nodules and not ulcerated ones (Barral et al., 1991; Silveira et al., 2004; Tolezano et al., 2007).

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Leishmaniasis prevention through prophylactic immunisation seems feasible (Grenfell et al., 2010). It is expected that an ideal leishmaniasis vaccine will combine more than one antigen, and that antigens will preferentially be conserved among *Leishmania* species and be present in both the amastigote and promastigote stages. Although several current candidates fulfill such criteria, a demonstration of the protection by these antigens in more than one animal model is lacking (de Oliveira et al., 2009; Grenfell et al., 2010).

The ultimate goal of vaccination is to generate a strong immune response, which will confer long lasting protection against leishmaniasis. Since subunit candidates and purified protein antigens are poor immunogens (Titus et al., 1995), they require the addition of an adjuvant or antigen-carrier system to be effective. The adjuvant should be selected according to features such as the employed antigens, the animals to be vaccinated, the administration route and the side-effects (Nagill and Kaur, 2010). Recently, technologies that incorporate adjuvants with the aim to increase the immunogenicity of antigens have been appointed as one of the top 10 technologies likely to significantly impact global health (Mutwiri et al., 2004). The use of nanotechnology proceedings have been applied in the design of new adjuvants and carriers for vaccine development in several areas, such as specific professional cell antigen delivery (Mutwiri et al., 2004; Badiee et al., 2007). Indeed, many results in literature demonstrated how liposomes are being employed as appropriate antigen carriers for the generation of antibody response *in vivo* and as

effective vehicles for peptides and proteins, thus enhancing their immunogenicity in leishmaniasis infection models (Uemura et al., 2005; Badiee et al., 2007; Mazumder et al., 2011; Shargh et al., 2012).

T cells recognise antigenic peptides derived from cytosolic proteins associated with MHC I molecules or antigenic peptides derived from the lysosomal compartment in association with the MHC II molecules on the antigen presenting cells (APCs) surface (Rao and Alving, 2000). It seems that almost any protein epitope might work as an antigen, regardless of the location in the parasite (Handman, 2001). It is well known that liposomes direct peptide antigens into the MHC II pathways of APCs, resulting in the enhancement of antibody and/or antigen-specific T-cell response based on the their formulations (Rao and Alving, 2000). The antigens released from these particles have proven to be a very effective delivery strategy for passive targeting to APCs by size exclusion mechanism, protection from protease degradation, cellular uptake enhancement, antigen depot formation at the injection site and controlling the release rates of antigens (Nguyen et al., 2009).

Since the host immune response against the infection with the parasite antigens depends on how they are presented to the effector cells of the immune system of the host, the reconstitution of membrane proteins in liposomes becomes a useful tool in the preparation of antigenic components to induce immunity in animal models (Mazumder et al., 2011; Shargh et al., 2012).

Here, we prepared an extract of GPI-anchored proteins from *L. amazonensis* promastigotes and reconstituted them into liposomes. These experiments were performed to characterise the process of proteoliposomes construction and this material employed to induce an immune response in BALB/c mice, being able to show significant protection from homologous infection. Our results with proteoliposomes indicate a possible application of these mimetic membrane systems in the preparation of a putative vaccine prototype against *L. amazonensis*.

Methods

Chemicals

All solutions were prepared using Millipore DirectQ ultra pure apyrogenic water. bovine serum albumin (BSA); dipalmitoylphosphatidylcholine (DPPC); 1-piperazineethane sulphonic acid; 4-(2-hydroxyethyl)-monosodium salt (HEPES); sodium dodecylsulphate (SDS); phosphate buffered saline (PBS); cholesterol, Triton X-114; Tris(hydroxymethyl)aminomethane (Tris); RPMI-1640 and Schneider's medium were purchased from Sigma Chemical Co. (St Louis, MO). Biobeads SM2-adsorbent resin was obtained from Bio-Rad (Hercules, CA). All other reagents, salts and solvents, p.a. grade, were acquired from known suppliers. RPMI-1640 medium was supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Cultilab, Brazil), 20 mM HEPES, 0.5 μg mL^{-1} gentamicin, and 0.2 μg mL^{-1} sodium bicarbonate.

Mice and parasites

Male or female BALB/c mice, 4–6 weeks old, were bred and maintained under standard conditions in the animal facility at Faculdade de Medicina de Ribeirão Preto, University of São Paulo or at Fiocruz Rondonia, Porto Velho, Rondonia.

The *L. amazonensis* parasite strain IFLA/BR/67/PH8 was maintained in BALB/c mice as previously described (Nunes et al., 1997; Noronha et al., 1998). The amastigotes were isolated from footpad lesions of infected mice and transferred to Schneider's medium. All experiments were approved and conducted in accordance with guidelines of the Animal Care Committee of the IPEPATRO/Fiocruz Rondonia (registration no 2008/005).

Preparation and solubilisation of *L. amazonensis* GPI-anchored protein

Leishmania amazonensis promastigotes were grown at 26 °C in Schneider's medium containing 10% FBS. Promastigotes were harvested at the early stationary phase (day 4–5), at the peak of their cytolytic activity (Noronha et al., 1996). Parasites were washed twice with 50 mM Tris-HCl buffer pH 7.25 containing 10 mM NaCl and 2 mM $CaCl_2$. Pellets obtained by centrifugation at 1200 × g for 15 min were kept at −20 °C until use. The frozen promastigote pellets were re-suspended in the same buffer and sonicated at 4 °C, for 30 s, at 240 W, followed by centrifugation at 1000 × g for sedimentation of organelles, nuclei and intact cells, which were discarded. Membrane fractions were isolated after supernatant ultracentrifugation at 100 000× g for 1 h, at 4 °C. The re-suspended pellet was ultracentrifuged again, in the same conditions above. Samples of membrane fraction proteins (0.5 mg/mL) were solubilised in 1% Triton X-114 for 1 h, at 4 °C, under constant agitation, and vortexed at 10-min intervals (Bordier, 1981). After phase separation at 20 °C, the detergent-rich phase (extract protein solubilised rich in GPI proteins – EPS_{GPI}), aqueous phase (poor in solubilised proteins) and membrane-containing pellet (not solubilised material) were frozen until use.

Estimation of protein concentration

Protein concentration was estimated in the presence of SDS 2.0% (w/v), following the procedure described by Hartree (1972), using crystallised BSA as standard.

Liposomes and proteoliposomes preparation

Liposomes and proteoliposomes were prepared using a 1.2:1 DPPC/cholesterol molar ratio. The phospholipids and cholesterol (6 mg) were dissolved in 1 mL chloroform and dried under nitrogen flow; the obtained lipid film was maintained under vacuum for 1 h. Next, 6 mL 50 mM Tris-HCl buffer solution was added to the film. The mixture was incubated for 1 h, at 60 °C, above the lipid critical temperature and vortexed at 10 min intervals. Then, the lipid emulsion was sonicated for 2 min, at 240 W, by means of a microtip. The obtained mixture was centrifuged at 100 000× g for 20 min, at room temperature, and the pellet was discarded. The supernatant, which corresponds to small unilamellar vesicles, was used to obtain the proteoliposome (Colhone et al., 2009).

A protein sample obtained from the Triton X-114-rich phase (0.25 mg total protein) was treated with 0.07 g/mL Biobeads hydrophobic resin for 5 h, at 4 °C, to remove excess detergent. The resulting sample was then mixed with 4 mL of the previously obtained liposome. The mixture was incubated at room temperature, overnight. Finally, the resulting solution was centrifuged at 100 000× g for 1 h. The pellet, constituted by proteoliposomes, was resuspended in 50 mM Tris-HCl buffer, and the supernatant was discarded. The phospholipids and cholesterol were quantified as previously described (Chen et al., 1956; Higgins, 1987).

Light scattering of liposomes and proteoliposomes

The determination of liposome and/or proteoliposome size distribution was carried out by dynamic light scattering (DLS) using a Beckman Coulter (model N5 submicron particle size analyser, Beckman Coulter, Inc., Fullerton, CA). The average value of the liposome diameters was obtained from the unimodal distribution. All samples were filtered and diluted to an adequate polydispersion index.

Isopycnic density gradient centrifugation

A continuous sucrose gradient was prepared (1–15% sucrose in 50 mM Tris-HCl buffer, density ranging from 3.0 to 10.6 g mL^{-1}) using a Gradient Maker (Hoefer Scientific Instruments, San Francisco, CA). Samples of solubilised GPI-anchored protein, liposomes or proteoliposomes (1 mL) were loaded onto the gradient and centrifuged in a Hitachi P65VT3 vertical rotor, in a Hitachi 55 P-72 ultracentrifuge at 180 000 × g for 4 h at 4 °C. The gradients were then fractioned into 0.5 mL fractions and analysed for protein and inorganic phosphate content, as described by Chen et al. (1956).

Antiserum preparation

BALB/c mice were infected subcutaneously in the right-hind footpad with 10^6 stationary growth phase *L. amazonensis* promastigotes. Two months after infection, the animals were bled, and the serum against total *L. amazonensis* antigenic determinants was collected. In addition, in order to analyse the protein extract of *L. amazonensis* in the proteoliposome, BALB/c mice were immunised intraperitoneally with 40 μg of proteoliposomes, and the serum was collected 14 weeks after immunisation.

SDS–polyacrylamide gel electrophoresis and Western blotting

Determination of the molecular mass of the 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 separating gel. The gels were silver stained for visualisation of the protein band profile. Molecular weight markers ranged from 10 to 120 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% (w/v) Tween 20) for 1 h and probed with antiserum against total *L. amazonensis* at a dilution of 1:75 in the blocking buffer overnight. Excess proteins were removed with four 5 min washes, with washing buffer (PBS, containing 0.05% (v/v) Tween 20). Finally, the membrane was probed with peroxidase-labelled goat anti-mouse IgG conjugate at a 1:1000 dilution in the washing buffer. The DAB substrate was used to reveal protein–antibody interaction.

Evaluation of protection against infection with *L. amazonensis*

Groups of five animals were intraperitoneally (i.p.) immunised with liposome, different amount of GPI-anchored protein extract solubilised with Triton X-114 (EPS_{GPI}), or of GPI protein incorporated in proteoliposome. The control group received Tris-HCl 50 mM. At the 3rd week after immunisation, mice were challenged with one subcutaneous injection with 1×10^4 stationary growth phase *L. amazonensis* promastigotes in the right hind footpad. Lesion development was monitored once a week using a caliper (125MEA-6/150; Starrett, São Paulo, Brazil). The lesion size was determined by subtracting the thickness of the uninfected contra-lateral footpad from the thickness of the infected one.

Statistical analysis

Data were reported as the mean (±SD) of five measurements of three different experiments and were submitted to variance (one-way ANOVA) analysis, followed by Turkey test, using the GraphPad Prism 5 software. Values were considered statistically significant when $p \leq 0.05$.

Results

Characterisation of liposome and proteoliposome

In order to prepare liposome and proteoliposomes, DPPC/cholesterol molar ratio (1.2:1) and 0.0625 mg mL^{-1} of Triton X-114 solubilised GPI-protein extract (EPS) were used. DLS technique can provide information on the size of the liposome system and it is very important in the study of the structural characteristics of vesicles in suspension. Thus, we determined the average particle size and a significant increase in liposome diameter size (about 60%) were observed after the incorporation of proteins. Empty liposomes exhibited a mean diameter size of 89 nm, while the proteoliposomes had an average diameter size of 220 nm. In addition, it is important comment that about 60% of the GPI-proteins were reconstituted in the proteoliposomes systems and these vesicles were very stables when stored one week 4 °C, since no significant diameters changes were observed.

To characterise the incorporation rate of *L. amazonensis* GPI-protein into proteoliposomes, an isopycnic density gradient centrifugation was performed, and the fractions were assayed for protein and phosphate contents (Figure 1). A peak for the inorganic phosphate, generated from the acid hydrolysis of the lipids, was found in the range of 4% sucrose, corresponding to the liposome fraction (Figure 1A). As shown in Figure 1(B), analysis of the solubilised GPI-protein extract revealed the presence of protein in the middle region of the gradient (around 8–10% sucrose concentration). The proteoliposomes construct showed a single peak at 6% sucrose, for both the

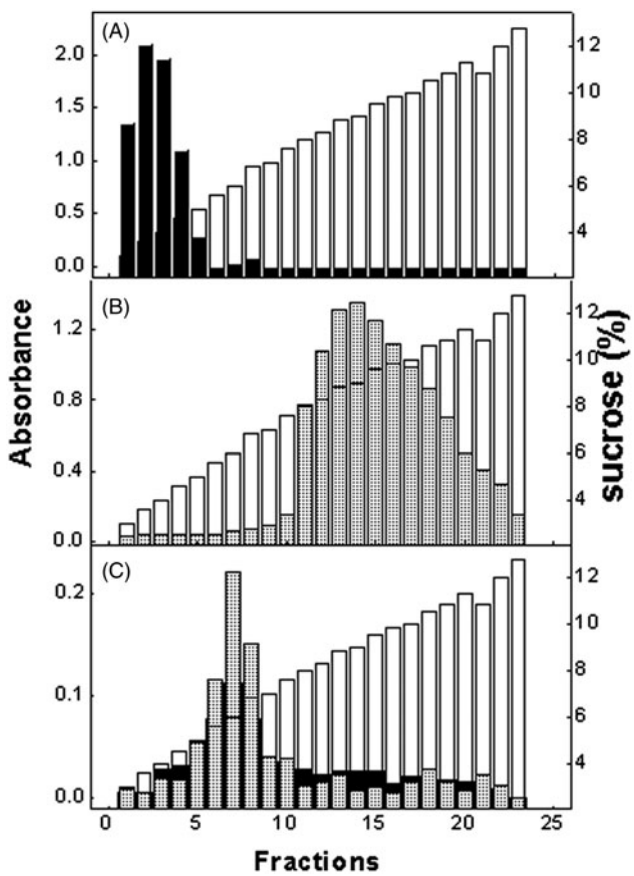

Figure 1. Isopycnic density sucrose gradient centrifugation study. (A) Liposome; (B) Triton X-114-solubilised protein extract from *L. amazonensis*; and (C) Proteoliposome (black symbol) lipid, absorbance at 820 nm; (gray symbol). Protein, absorbance at 595 nm and (white symbol) percent sucrose.

inorganic phosphate and protein, indicating efficient incorporation during reconstitution of the solubilised GPI-protein extract (Figure 1C).

In addition, SDS-PAGE of proteoliposomes revealed a variety of proteins with a distribution similar to that observed in the Triton X-114-solubilised protein extract of *L. amazonensis*, indicating the nonselective incorporation of parasite proteins into the liposomes (Figure 2A). Moreover, Figure 2(B) shows the antigenic profiles of the crude extract, Triton X-114-solubilised protein extract and proteins incorporated in proteoliposomes after reaction with antiserum against total *L. amazonensis* antigenic determinants. Note that the protein reconstitution in liposomes is not proportional for each protein present in EPS_{GPI}. All solubilised proteins present in the EPS extract did not incorporate in liposomes with the same proportion present in the membrane. As a consequence, the Western blots show with greater intensity a protein that was enriched in the proteoliposome, and this different constitution could be important in the immunisation process, as shown in the sequence.

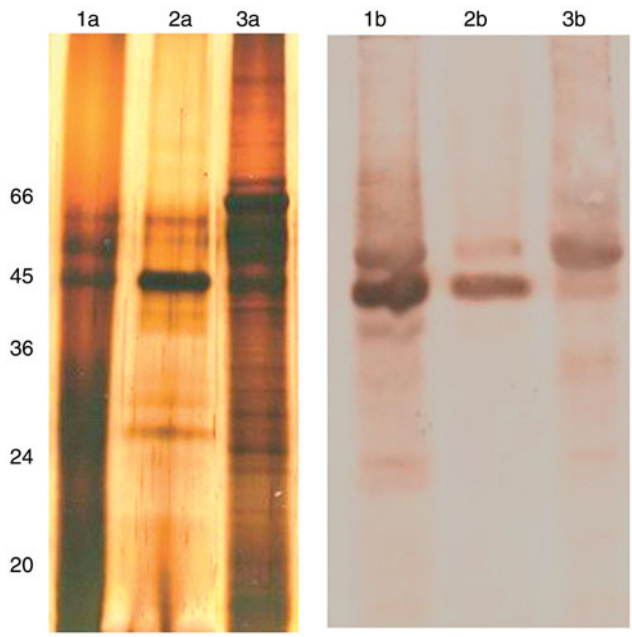

Figure 2. SDS-PAGE gel proteins and Western blotting from promastigotes *L. amazonensis* membrane proteins. (A) Silver stained proteins and (B) Western blotting of lanes: (1) membrane fractions; (2) solubilised proteins extract (EPS) and (3) protein reconstituted in the proteoliposomes, as described in 'SDS–polyacrylamide gel electrophoresis and Western blotting' section.

Evaluation of infection blockage with *L. amazonensis* BALB/c

To evaluate whether proteoliposomes-carrying *L. amazonensis* antigens could induce immune response in BALB/c mice, animals were immunised with either Tris-HCl buffer (50 mM) control, liposomes, 10 and 20 μg EPS_{GPI}/animal, or 120 μg of total protein present in proteoliposomes/animal. These proportions were always related to the amount of protein present in the samples. Mice received one immunisation, and three weeks later the animals were challenged by subcutaneous injection of 10^4 stationary growth phase *L. amazonensis* promastigotes into the right-hind footpad. It has been verified that BALB/c mice infected with *L. amazonensis* displayed progressive lesions, which reached maximum size at 14 weeks post-infection. Mice inoculated with 5, 10 or 20 μg of total protein present in proteoliposomes showed no significant differences in the size of the footpad lesion (Figure 3). However, as depicted in Figure 4, mice immunised with 40 μg of total protein present in proteoliposomes showed partial protection (about 50%) very similar with 5 μg EPS_{GPI} showed greater ability to induce partial protection (60%), as compared to control group (Figure 4).

Meanwhile, mice inoculated with 120 μg of total protein present in proteoliposomes presented significant reduction ($p \leq 0.05$) in the size of the footpad lesion (about 90%), as shown in Figure 5, suggesting a synergic effect of the microencapsulation system. Interestingly, 10 and 20 μg EPS_{GPI} was also able to significantly reduce ($p \leq 0.05$) the size of the footpad lesion (about 70%), as compared to control group (Figure 5).

Although empty liposomes prompted a residual protection in mice immunised with this system, this protection was significantly lower when compared to animals immunised with 120 μg of total protein present in proteoliposomes or with 10 or 20 μg EPS_{GPI} (Figure 5).

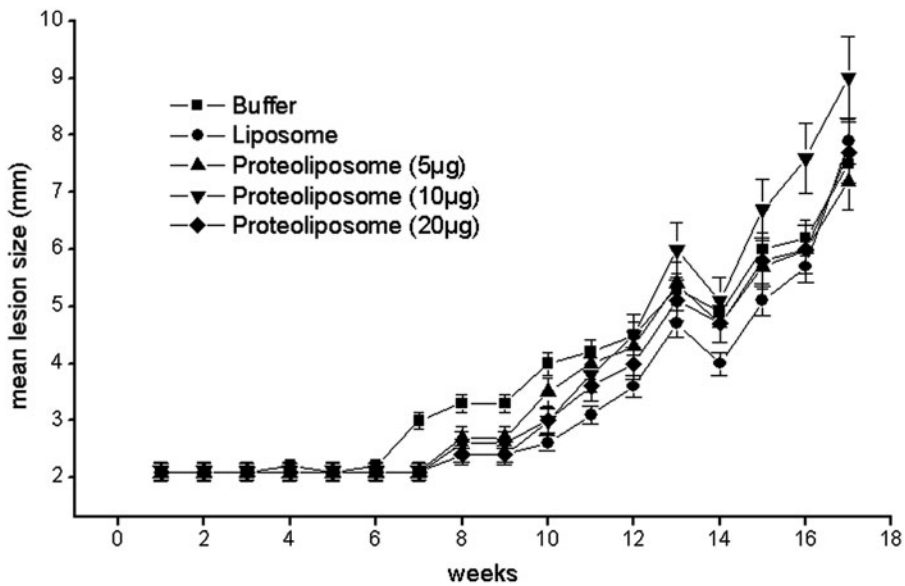

Figure 3. Evaluation of protection against infection with *L. amazonensis* – Groups of five animals were immunised i.p. with liposome or proteoliposomes (5, 10 or 20 μg of total protein/animal). The control group received Tris-HCl 50 mM (buffer). At the 3rd week after immunisation, mice were challenged with one subcutaneous injection in the right hind footpad with 1 x 10^4 stationary growth phase *L. amazonensis* promastigotes. Footpads were measured weekly with a caliper and the lesion size was determined by subtracting the thickness of the uninfected contra-lateral footpad from that of the infected one.

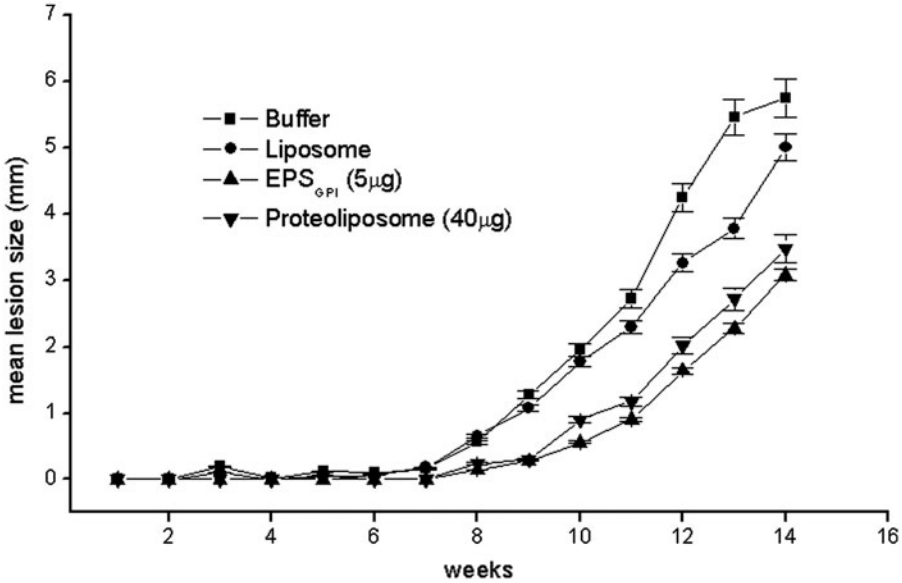

Figure 4. Evaluation of protection against infection with *L. amazonensis* – Groups of five animals were immunised i.p. with liposome, 5 μg of GPI-anchored protein solubilised extract by Triton X-114 (EPS_{GPI}) per animal and proteoliposomes (40 μg of total protein/animal). The control group received Tris-HCl 50 mM (buffer). At the 3rd week after immunisation, mice were challenged with one subcutaneous injection in the right hind footpad with 1×10^4 stationary growth phase *L. amazonensis* promastigotes. Footpads were measured weekly with a caliper and the lesion size was determined by subtracting the thickness of the uninfected contra-lateral footpad from that of the infected one.

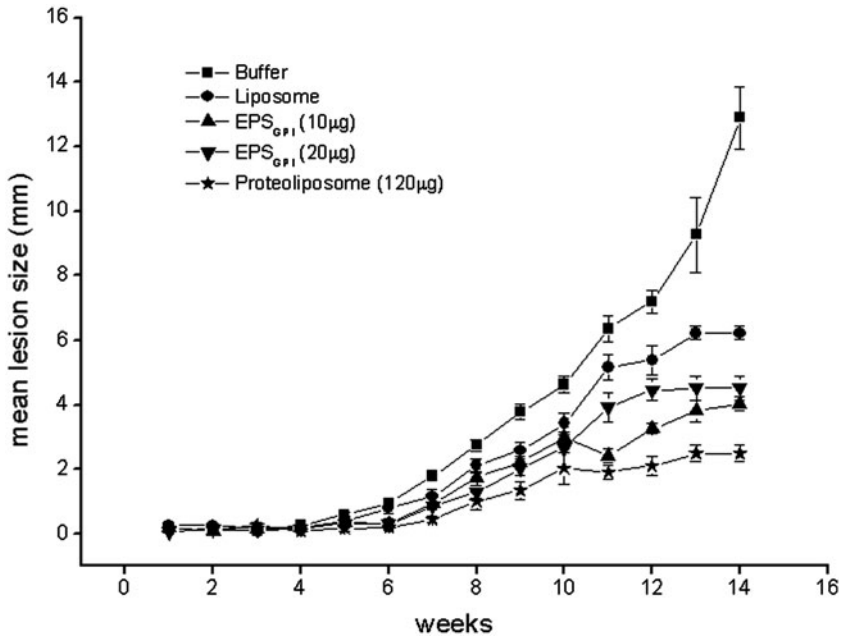

Figure 5. Evaluation of protection against infection with *L. amazonensis* – Groups of five animals were immunised i.p. with liposome, 10 or 20 μg of GPI-anchored protein solubilised extract by Triton X-114 (EPS_{GPI}) per animal and proteoliposome (120 μg of total protein/animal). The control group received Tris-HCl 50 mM (buffer). At the 3rd week after immunisation, mice were challenged with one subcutaneous injection in the right hind footpad with 1×10^4 stationary growth phase *L. amazonensis* promastigotes. Footpads were measured weekly with a caliper and the lesion size was determined by subtracting the thickness of the uninfected contra-lateral footpad from that of the infected one.

Discussion

Our data concerning the use of sucrose density gradients to characterise protein incorporation on the lipid bilayer of liposomes suggest that liposomes reconstituted with DPPC and cholesterol present a peak of phosphate due to the acid hydrolysis of phospholipids (Daghastanli et al., 2004; Santos et al., 2006). This essay shows evidence of liposome populations distributed in a single peak, indicating the presence of homogeneous liposomes populations. Thus, 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. Furthermore, these systems appear in lower densities probably because of their cholesterol content, as described by other authors (Cevc and March, 1987).

In addition, SDS-PAGE of proteoliposomes revealed a variety of proteins with a distribution similar to that observed in the Triton X-114-solubilised protein extract of *L. amazonensis*, indicating the nonselective incorporation of parasite proteins into the liposomes. Thus, our results clearly show that the method employed here may lead to efficient membrane-protein reconstitution and suggest that *L. amazonensis* GPI-proteins are efficiently incorporated on proteoliposomes without affecting their immunogenicity.

The parasites plasma membrane plays an important part in the infection processes. Indeed, cell surface proteins and glycoproteins display a relevant structural role and are involved in different cell mechanisms such as adsorption, nutrient transport, cell recognition and escape (De Souza, 1995). Maybe these proteins' sprayed functions make them a target for an effective immune response and that is why they have been widely employed in the preparation of vaccines.

In recent years, several efforts have been made toward the attainment of a safe and efficient vaccine against leishmaniasis. Vaccination with live, attenuated parasites has been attempted (Streit et al., 2001; Nylén et al., 2006), although there are several ethical considerations regarding of these vaccines methodologies. Probably, the human vaccine prototype will require several

different antigens (extracted or recombinant) and/or adjuvants, in order to guarantee a satisfactory vaccine response, given the population heterogeneity (Handman, 2001).

In this sense, a successful approach has been the isolation of compounds, particularly proteins, from the membrane of pathogens, followed by their reconstitution in liposomes (Daghastanli et al., 2004; Santos et al., 2006; Jaafari et al., 2007). These processes enable some structural particularities preservation, which are necessary for the development of antigenicity. It has been demonstrated that the original conformation of antigen is important for the best processing and epitope presentation to the T cell (Janssen et al., 1996). Reconstitution of membrane proteins in liposomes has proven to be a viable alternative for the preparation of antigenic components that induce immunity (immunogenicity) in animal models (Leserman, 2004). In addition to conserving the native structure of proteins, liposomes have the advantage of providing adjuvant activity in vaccines against various pathogens (Daghastanli et al., 2004; Santos et al., 2006; Laing et al., 2006).

Although empty liposomes prompted a residual protection in mice immunised with this system, this protection was significantly lower when compared to animals immunised with proteoliposomes or with EPS_{GPI}. Perrie et al. (2013) have confirmed that cationic liposomes formed a depot at the injection site, which stimulates recruitment of APCs to the injection site and promotes strong humoral and cell-mediated immune responses. Physicochemical factors, which promote a strong vaccine depot, include the combination of a high cationic charge and electrostatic binding of the antigen to the liposome system and the use of lipids with high transition temperatures, which form rigid bilayer vesicles. Reduction in vesicle size of cationic vesicles did not promote enhanced drainage from the injection site. However, reducing the cationic nature through substitution of the cationic lipid for a neutral lipid, or by masking of the charge using PEGylation, resulted in a reduced depot formation and reduced Th1-type immune responses, while Th2-type responses were less influenced. These studies confirm that the physicochemical characteristics of particulate-based adjuvants play a key role in the modulation of immune responses (Perrie et al., 2013).

Some studies have described that antigens alone are generally weak immunogens (O'Hagan et al., 2001) and require an adjuvant for induction of protective immunity (Nagill and Kaur, 2010). However, the quality of the generated immune response depends on the combined action of the antigen and the adjuvant and the physical nature of the liposomes (Felnerova et al., 2004; Bhowmick et al., 2007). Nagill and Kaur (2010) have observed that immunisation with purified native 78 kDa antigens alone or along with different adjuvants conferred significant protection against a progressive infection with *L. donovani* in mice. Nevertheless, the degree of protection varied with the adjuvant employed in vaccine formulations.

A widely studied protein that can be a possible target for vaccines is gp63, which is involved in several events related to host–parasite interactions. Jaafari et al. (2006) have shown that the growth of the footpad lesion in BALB/c mice immunised with recombinant gp63 (rgp63), associated with liposomes, decreased significantly after challenge with *L. major* promastigotes. Interestingly, the growth of the footpad partly diminished in mice immunised with rgp63 alone (Jaafari et al., 2007).

Immunisation of BALB/c using the native protein gp63 purified and incorporated into liposomes significantly protected all the animals against progressive infection with *L. donovani* (Bhowmick et al., 2008; Mazumder et al., 2011). Similarly, total soluble antigens of *L. donovani* (Ali and Afrin, 1997; Afrin et al., 2002; Mazumdar et al., 2004; Bhowmick et al., 2007) and *L. major* (Shargh et al., 2012) promastigotes and pDNA encoding cysteine proteinase type II (CPA) and type I (CPB) (Doroud et al., 2011) associated with liposomes have also been used for mouse immunisation. In these systems, high protection levels (70–87%) against infection with the parasite have been reached. Indeed, there are many studies using parasites surface proteins associated with liposomes, and high protection rates have been reported. Still, Brewer et al. (1998) indicated that lipid vesicles with a mean diameter >225 nm preferentially induce Th1 responses in BALB/c mice. Yuba et al. (2010) investigated the feasibility of polymer-modified liposomes as a carrier of antigenic proteins for induction of cellular immunity and observed that these liposomes were taken up efficiently by cells, thereafter delivering their contents into the cytosol, probably through fusion with endosomal membranes. Murine bone marrow-derived dendritic cells (DCs) treated with polymer-modified liposomes encapsulating OVA stimulated CD8-OVA1.3 cells more strongly than OT4H.1D5 cells, indicating that the liposomes induced MHC class I-restricted presentation (Yuba et al., 2010).

Moreover, liposomes represented a promising vehicle to deliver exogenous antigens to DCs for tumour immunotherapy, as described by Kawamura et al. (2006) that demonstrated an IgG liposome with physicochemical properties is suitable for delivering antigens to DCs and pave the way to the application of IgG liposomes for tumour immunotherapy using DCs.

However, there are no published data on the protection of susceptible mice using a pool of GPI-anchored proteins from the surface of promastigotes of *L. amazonensis* associated with liposomes consisting of DPPC/cholesterol, as in the case of the present study. Furthermore, the GPI-anchored proteins employed in our work proved to be strong immunogens and induced partial protection (70%) in the absence of adjuvant in immunised mice that were subsequently challenged with the parasite. Other authors have also observed a similar behaviour. Soluble antigens in the absence of adjuvants were immunogenic, which resulted in partial protection, and suggested that these molecules may have inherent adjuvant activity (Ali and Afrin, 1997; Jaafari et al., 2006). Based on all these data, we suggest that the development of protein-based vaccination could be greatly influenced by using suitable and effective adjuvant systems that promote the slow release of antigens at the site of immunisation with their ability to trigger the immune system.

Conclusion

Mice inoculated with EPS_{GPI} and total protein present in constitutive proteoliposomes exhibited a protection of about 70% and 90%, respectively, post-infection. Therefore, our results have indicated that either EPS_{GPI} or proteoliposomes, carrying GPI proteins may be used to induce protective immunity against *L. amazonensis* in mice. Therefore, these systems seem to be promising vaccine systems for immunisation against leishmaniasis.

Declaration of interest

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